Oxygen-Dependent Regulation of Hypoxia-Inducible Factor (HIF) and its Role in Cancer Therapy Resistance

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Meinen Eltern

für ihre Unterstützung und Liebe

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List of abbreviations

AAG	17-(allylamino)-17-demethoxygeldanamycin
AE	anion exchanger
AhR	aryl hydrocarbon receptor
Akt	protein kinase B
ARNT	aryl hydrocarbon receptor nuclear translocator
Asn	asparagine
ATM	ataxia-telangiectasia mutated
ATR	ATM- and rad3-related
ATRIP	ATR interacting protein
Bad	BCL2-antagonist of cell death
BAX	BCL2-associated X protein
BCA-assay	bicinchoninic acid assay
BCL2	B-cell leukemia/lymphoma 2
bHLH	basic helix-loop-helix
BID	BH3 interacting domain death agonist
BLM	Blooms syndrome homolog (helicase)
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein3
BRCA1	breast cancer 1 (tumor suppressor gene)
BRCA2	breat cancer 2 (tumor suppressor gene)
CAIX	carbonic anhydrase 9
CBP	CREB (cAMP-responsive element binding protein) binding protein
cdc25	cell divison cycle mutant 25
cDNA	complementary DNA
CDKI	cyclin-dependent kinase inhibitor
Chk1	checkpoint homolog 1
Chk2	checkpoint homolog 2
CPX	ciclopirox olamine
DAPI	4',6-Diamidino-2-phenylindol
DFX	desferroxamin
DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
DMOG	dimethyloxalyl glycin
DNA	deoxyribonucleic acid
DNA-PK	DNA-activated protein kinase,
DSB	double-strand breaks
DTT	dithiothreitol
ECL	enhanced chemiluminescent
EDTA	ethyl-diamine-tetra-acetate
EGLN	egg laying deficiency
ErbB2	oncogene, receptor tyrosine kinases
FAT	protein interaction domain (FRAT, ATM, TRAPP)
FATC	protein interaction domain (FRAT, ATM, TRAPP, C-terminal)
FACS	fluorescence activated cell scanner
FAK	focal adhesion kinase
FGF	fibroblast growth factor

FH	fumarate hydratase (tumor suppressor)
FIH	factor inhibiting HIF
FKBP	FK506-binding protein
GA	geldanamycin
GADD	growth arrest and DNA damage
GLUT	glucose transporter
GST	glutathione-S-transferase
HEAT	protein interaction domain (huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR1)
HeLa	cervix carcinoma cell line (patient name: Henrietta Lacks)
Hepa	hepatoma cell line
HIF	hypoxia inducible factor
HPH	HIF prolyl hydroxylase
HR	homologous recombination repair
HRE	hypoxia response element
hSMG-1	suppressor of morphogenesis in genitalia-1
HSP	heat shock protein
H2A	histone 2A
H2AX	histone 2A sub-variant X
ING4	inhibitor of growth family, member 4
IR	ionizing radiation
Ku70	thyroid-lupus autoantigen p70
Ku80	thyroid-lupus autoantigen p80
MAPK	mitogen activated protein kinase
Mcm4	minichromosome maintenance-deficient 4 homolog
MDR1	multidrug resistance 1
MEF	mouse embryonic fibroblast
Mre11	meiotic recombination 11
MRN	Mre11/Rad50/Nbs1
mTOR	mammalian target of rapamycin
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
NBC	sodium-bicarbonate co-transporter
Nbs	Nijmegen breakage syndrome
NHE	Na^+/H^+ exchanger
NHEJ	non-homologous end-joinging
N-OG	N-oxyalylglycine
ODDD	oxygen dependent degradation domain
OS-9	amplified in osteosarcoma
PAS	Per-ARNT-SIM
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEI	polyethylenimine
PHD	prolyl hydroxylase domain
PIKK	phosphatidyl inositol kinase related kinase

pRB	protein retinoblastoma
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PVDF	polyvinylidene difluoride
pVHL	protein von Hippel-Lindau (tumor suppressor protein)
p107	protein 107, pocket protein analog to pRB
p130	protein 130, pocket protein analog to pRB
p16/INK4a	protein 16/inhibitor of kinase 4a (cell cycle inhibitor)
p21waf1/cip	protein 21 (wild-type p53 fragment 1/cell-cycle inhibitor protein)
p27 kip	protein 27 (cell-cycle kinase inhibitor protein)
p300	protein 300 (histone acetyl transferase)
p53	protein 53, „master guardian of the cell“
qPCR	quantitative PCR
RACK1	receptor of activated protein kinase C 1
ras	rat sarcoma (proto-oncogene)
RNA	ribonucelic acid
RNAi	RNA inhibition
ROS	reactive oxygen species
RPA	replication protein A
R point	restriction point of the cell cycle
RTK	receptor tyrosine kinase
Rad50	DNA repair protein
Rad51	DNA repair protein
Rad52	DNA repair protein
Rad54	DNA repair protein
RT-qPCR	reverse transcription-qPCR
SDH	succinate dehydrogenase (tumor suppressor)
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
Siah2	seven in absentia homolog 2
siRNA	small inhibitory RNA
src	simian rous sarcoma (proto-oncogene, tyrosine kinase)
ssDNA	single-stranded DNA
SV40	simian virus
TAD	trans activation domain
[N-TAD or C-TAD	N –terminal or C-terminal TAD]
TCF3	transcription factor 3
TdT	terminal deoxynucleotidyl transferase
TFG	transforming growth factor
TLC	thin layer chromatography
TRiC	TCP ring complex chaperonin
Tris-HCl	Tris (hydroxymethyl) aminomethane Hydrochloride

TRRAP	transformation/transcription domain-associated protein
UV	ultraviolet (λ=100-400 nm)
VBC	pVHL/elongin B/elongin C
VEGF	vascular endothelial growth factor
VP-16	vepeside 16 (etoposide)
v-src	viral-src
WRN	Werner syndrome homolog (helicase)
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
YC-1	3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole
53BP1	p53 binding protein

Zusammenfassung

In einem Organismus sind zahlreiche Regulierungssysteme aktiv, um den sich ständig wechselnden Umwelteinflüssen zu begegnen und so ein Überleben zu ermöglichen. Anpassungen an die jeweilige Situation erfordern dabei sowohl die Regulation auf der Stufe Organismus als auch auf Stufe Zelle. Im Zentrum des zellulären Regulierungssystems, welches die Anpassung an eine Sauerstoffmangel-Situation (Hypoxie) bewerkstelligt, steht der Hypoxie-induzierbare Transkriptionsfaktor (HIF). HIF ist ein heterodimerer Transkriptionsfaktor bestehend aus einer nicht regulierten β-Untereinheit und einer regulierten, labilen α-Untereinheit. Als Zellantwort auf eine Hypoxie erfolgt eine verminderte Hydroxylierung der labilen α-Untereinheit. Dies führt zu einem Unterbruch des proteasomalen Abbaus der labilen α-Untereinheit und daher zur Akkumulation von HIFα, was mit einer erhöhten Expression von HIF-Zielgenen einhergeht. Die Hydroxlierung an HIFα vermittelt so auf elegante Weise einen direkten Zusammenhang zwischen Sauerstoffkonzentration und HIF abhängiger Transkription, wobei die für die Hydroxylierung verantwortlichen Enzyme, die Prolyl-4-Hydroxylasen (PHD), als eigentliche Sauerstoffsensoren fungieren. Wir betrachteten deshalb die Regulation und Aktivtät der PHDs genauer, um die bekannte Korrelation von erhöhtem HIFα mit Tumortherapie-Resistenz und Tumorprogression aufzuklären. Tatsächlich etablierten *in vitro* Versuche einen „feed-back loop“, wodurch basale Mengen von HIFα dem vorherrschenden Sauerstoffpartialdruck angeglichen werden. Eine Störung der basalen Mengen von HIFα in Tumoren könnte zu einem schlechteren Behandlungserfolg des Krebses führen. So haben wir, beim Vergleich von HIFα defizienten (*Hif1a*$^{-/-}$) mit unveränderten (*Hif1a*$^{+/+}$) Maus Fibroblasten (MEF) unter Normoxie (d.h. basale Mengen von HIF) für mutante MEF-*Hif1a*$^{-/-}$ eine erhebliche grössere Empfindlichkeit gegenüber DNA Doppelstrang brechenden Chemotherapeutika (z.B. Etoposid) festgestellt – korrespondierend zu einer erhöhten Zahl von DNA Doppelstrangbrüchen nach Etoposidebehandlung. Beim Vergleich der mRNA Mengen von MEF-*Hif1a*$^{+/+}$ und MEF-*Hif1a*$^{-/-}$ Zellen für bekannte DNA Reparatur Proteine, stiessen wir

auf einen signifikanten Unterschied in der Menge von DNA-PK mRNA. DNA-PK ist eine Kinase mit zentraler Funktion im Nicht-Homologen End-joining (NHEJ) einem Hauptmechanismus der zellulären DNA Doppelstrangbruch Reparatur. Zudem sind auch die Proteinmengen des trimeren DNA-PK Komplexes drastisch tiefer in den mutanten MEF-*Hif1a*$^{-/-}$. Basale Mengen von HIF-1α scheinen daher die Konzentration der DNA-PK und damit auch die Reparaturkapazität einer Zelle zu beeinflussen, was in einer HIF-abhängigen Tumortherapie-Resistenz resultiert.

Summary

Tissue homeostasis is strictly controlled by numerous regulatory systems that are occupied with balancing the ever changing environmental conditions to ensure viability of the cell associations. This involves changes on the organismic as well as on the cellular level. The regulatory system which modulates the oxygen demand of a cell under oxygen limiting conditions vastly depends on the transcriptional activity of the hypoxia-inducible factor (HIF).

The transcriptional active form of HIF consists of a heterodimer with a non-regulated β- and a labile α-subunit. Upon hypoxic exposure, hydroxylation of the constitutively synthesized α-subunit decreases, thereby proteasomal HIFα degradation ceases and HIFα accumulates, leading to enhanced HIF-target gene expression.

The hydroxylation reaction of HIFα is governed by prolyl hydroxylases (PHDs) which depend on molecular oxygen (O_2) for activity and thus represent the sensory system that translates the O_2 partial pressure (pO_2) into the respective HIF-dependent gene expression profile.

In order to research the well-known correlation between HIFα levels, tumor progression and cancer therapy resistance, we examined the activity and regulation of the PHDs. *In vitro* PHD assays established a feed-back loop that might set basic levels of HIFα to the respective pO_2.

The deregulation of basic levels of HIFα in cancerous tissues could contribute to poor clinical therapy outcome. We found mouse embryonic fibroblasts (MEFs) deficient for HIF-1α (mutant MEF-*Hif1a*$^{-/-}$) to be more sensitive to double-strand inducing chemo-therapeutics (e.g. etoposide) than wild-type MEF-*Hif1a*$^{+/+}$ under normoxia (room air oxygen concentration) that is basic level function of HIF.

We could show that this corresponds to higher accumulation of double-strand breaks (DSB) after etoposide treatment. Furthermore, we compared mRNA expression levels of enzymes involved in DSB-repair for wild-type MEF-*Hif1a*$^{+/+}$ and mutant MEF-*Hif1a*$^{-/-}$. Hence, we discovered that the expression of DNA-PK, the primary kinase in non-homologous end-joining (a major DSB-

repair pathway), is significantly lowered in mutant MEF-*Hif1a*$^{-/-}$ cells when compared to wild-type MEF-*Hif1a*$^{+/+}$. In addition, also protein levels of the timeric DNA-PK complex are decreased in the mutant MEF-*Hif1a*$^{-/-}$ cell line. Thus, basic levels of HIF-1α may influence DNA-PK levels and dictate cellular DSB repair capacity that results in a HIF-dependent therapy resistance of cancerous tissues.

1 Introduction

1.1 The hypoxia-inducible factor (HIF)

HIF is a heterodimeric transcription factor which becomes stabilized after exposal to low oxygen conditions and thereafter induces expression of a distinct set of genes. It consists of a constitutively expressed subunit called HIFβ (also partner to the dioxin receptor/aryl hydrocarbon receptor (AhR), therefore also called AhR nuclear translocator (ARNT)) and an oxygen-regulated subunit called HIFα. The labile α-subunit is constitutively transcribed and translated but immediately degraded under normoxic conditions. In addition to HIF-1α two paralogues of the α-subunit (HIF-2α, HIF-3α) are known, each with a more restricted tissue distribution than the ubiquitously expressed HIF-1α (Wenger 2002).

Sequence comparison reveals that the two subunits (α, β) belong to the same family of basic–Helix-Loop-Helix-Per-ARNT-SIM (bHLH-PAS) proteins. The HLH and PAS motifs are required for dimer formation while the basic region mediates specific binding to DNA via short stretches of nucleotides encoding for hypoxia response elements (HREs, 5`-RCGTG-3`) in the vicinity of HIF target genes (Wang *et al.* 1995; Yang *et al.* 2005).

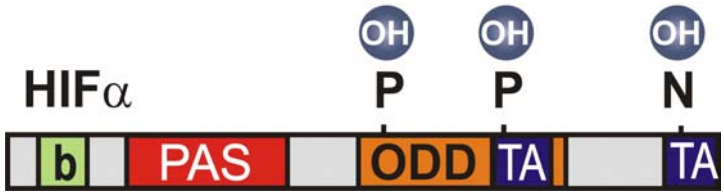

Figure 1. Outline of HIFα: regions/ domains are depicted as followed: b (=bHLH) [green], PAS [red], ODD [orange], N-TAD and C-TAD [blue] – P indicates a proline residue, N indicates an asparagine residue and OH reflects hydroxylation.

Other important regions - next to the dimerization and DNA binding domains - are the oxygen-dependent degradation (ODD) domain necessary for oxygen-regulated stability and two transactivation domains (TAD), named after their relative localization in the amino acid sequence of the protein: N-terminal or

C-terminal transactivation domain, N-TAD or C-TAD, respectively (Huang *et al.* 1998) (Fig.3).

1.2 Regulation of HIF activity

HIFα accumulation and activity is tightly coupled to the pO_2. In normoxia HIFα is immediately hydroxylated on proline 402 and/or 564 which form the conserved consensus sequence LXXLAP located in the ODD domain (Dann and Bruick 2005). This leads to von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex interaction. pVHL recognition in turn leads to poly-ubiquitination of HIFα and thereby marks it for degradation by the 26S proteasome (Maxwell *et al.* 1999; Kaelin 2005). Another post-translational modification used to restrict HIFα activity under high oxygen partial pressure includes hydroxylation of an asparagine residue in the C-TAD which prevents association of HIFα with the common transcriptional co-activators CBP and p300 (Hirota and Semenza 2005) (Fig.4).

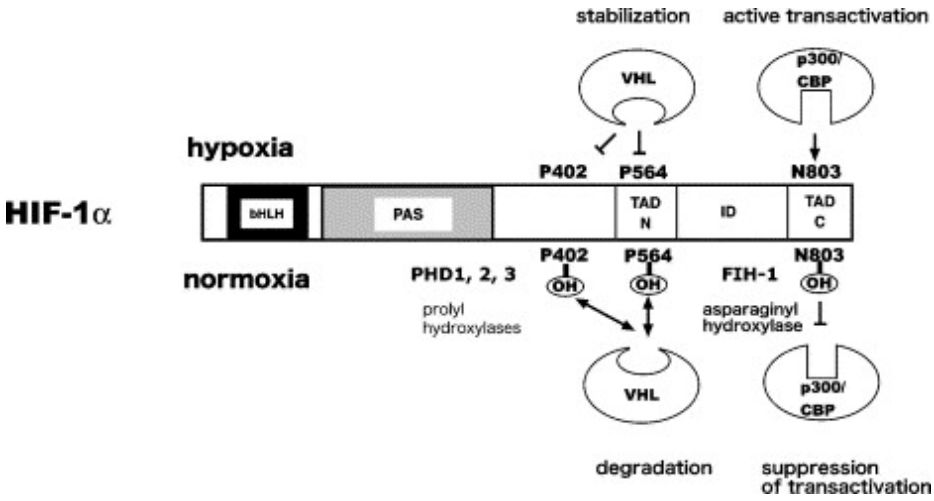

Figure 2. Regulation of HIFα under normoxic as well as hypoxic conditions. pVHL binding leads to ubiquitination and subsequent degradation of the protein. The prolyl hydroxylases are responsible for hydroxylation on specified proline residues. The hydroxylation is necessary for pVHL to interact. FIH hydroxylation prevents co-factor binding and thus suppresses transactivation of HIF-target genes (figure taken from Hirota and Semenza 2005).

Both hydroxylation events are performed by enzymes belonging to the family of 2-oxoglutarat-dependent dioxygenases (Aravind and Koonin 2001; Hausinger 2004). Whereas the enzyme controlling asparagine hydroxylation and co-activator recruitment is called factor inhibiting HIF (FIH) (Mahon *et al.* 2001), the oxygenases involved in HIF degradation by targeting the proline residues in the ODD domain are named prolyl-4-hydroxylase domain (PHD) proteins (Bruick and McKnight 2001; Schofield and Ratcliffe 2004).

The reaction performed by the 2-oxoglutarat-dependent dioxygenases adds one atom of molecular oxygen to a specific amino acid residue while oxidizing by the second oxygen atom 2-oxoglutarat, which in turn decarboxylates, forming carbon dioxide (CO_2) and succinate. An iron atom in the catalytic center of the enzymes serves as catalyst during the redox reaction as intermediate oxygen binding partner and by mediating the electron transfer. The requirement for ascorbate (vitamin C) in the reaction remains enigmatic but seems to be an at least catalytically important factor for enzyme function (Schofield and Ratcliffe 2004; Dann and Bruick 2005; Hirota and Semenza 2005) (Fig.5).

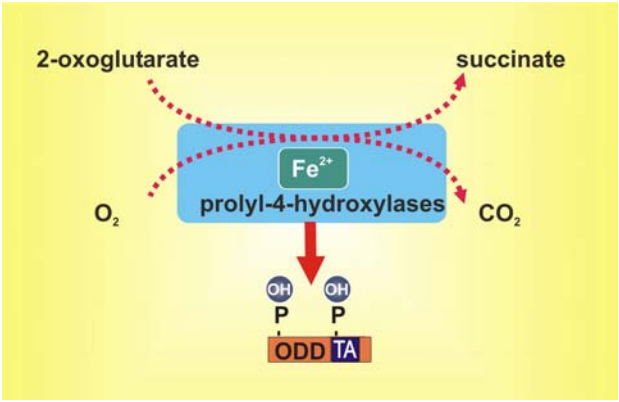

Figure 3. Scheme for the reaction performed by the prolyl-4-hydroxylases. The reaction is catalyzed by iron in the active center of the enzymes. Apart from hydroxylated proline residues carbon dioxide and succinate are produced. The substrates for the reaction are oxygen and 2-oxoglutarat. Ascorbate (not shown here) is catalytically necessary to complete the enzymatic reaction.

Thus, the absolute dependence of the dioxygenases on molecular oxygen links HIF activity with oxygen availability. Moreover, the reported Km (Michaelis-Menton) constant for all of the dioxygenases involved in HIF

activity regulation is higher than the molar concentration of oxygen dissolved in water at atmospheric pressure. Hence, enzyme activity is proportional to oxygen concentration and small deviations in the concentration of oxygen might affect enzyme activity linearly (Schofield and Ratcliffe 2005).

FIH dictates HIF activity by controlling transcriptional co-factor (CBP/p300) binding to the heterodimer. However, stability of HIF is achieved largely by preventing prolyl hydroxylation, hence placing PHDs into the center of HIF regulation (Schofield and Ratcliffe 2004).

The PHDs consist of three family members also known as HIF prolyl hydroxylases (HPH) 3, 2 and 1, or egg laying defective nine homolog (EGLN) 2, 1 and 3, respectively (Bruick and McKnight 2001; Epstein *et al.* 2001; Ivan *et al.* 2002). The enzymatic properties of the three different enzymes have been widely investigated and found to be comparable for hydroxylation efficiency of a synthetic peptide containing the consensus sequence and for Km values of the co-substrates (Hirsila *et al.* 2003; Oehme *et al.* 2004; Tuckerman *et al.* 2004; McNeill *et al.* 2005; Wirthner *et al.* 2007) (Tab.1).

Hydroxylase	K_m	
	O_2 (μM)	2-oxoglutarate (μM)
PHD1	250	60
PHD2	230	60
PHD3	230	35
FIH	90	25

Table 1. Km values for co-substrates. Whereas the PHDs have comparable values for oxygen the FIH differs. This might result in stable but transcriptional inhibited HIF.

More discrepancy between the enzymes is observed in their tissue distribution. Whereas PHD1 is most abundant in testis and brain, PHD3 expression is most prominent in the heart and PHD2 is found to be ubiquitously expressed (Soilleux *et al.* 2005; Stiehl *et al.* 2006). Likewise, also the intracellular distribution of the PHDs appears to be different. PHD1 seems to be restricted to the cytosol and PHD3 to the nucleus. PHD2 is detected

equally in both compartments (Metzen *et al.* 2003; Ke and Costa 2006). Interestingly, the response to hypoxia includes up-regulation of the PHD2 and PHD3 mRNA in a HIF-dependent way – with a pronounced PHD3 response (Stiehl *et al.* 2006). PHD1 is not induced by hypoxia. However, Siah2, a RING finger E3 ubiquitin ligase, targets PHD1 and PHD3 protein, adding another layer to HIFα regulation (Nakayama *et al.* 2004).

The three PHDs share 42-59% sequence identity but no similarity to the collagen prolyl-4-hydroxylases. Structurally, the most unique of the three oxidoreductases is PHD3 with a molecular mass of only around 27 kDa compared to 45 kDa and 47 kDa for PHD1 and PHD2, respectively (Hirsila *et al.* 2003). However, PHD2 contains an additional zinc finger domain located in the first exon. X-ray crystallographic analyses of FIH revealed the typical jelly-roll motif of eight β-strands folded into a double-stranded helix that is common for the 2-oxoglutarat dependent oxygenases. This motif comprises the three iron binding ligands which form a conserved two-histidine-one-carboxylate iron coordinating triad (Schofield and Ratcliffe 2004; Dann and Bruick 2005).

1.2.1 Oxygen-independent HIFα stabilization

Obviously, a large part of HIFα stabilization under normoxic conditions could be achieved by interfering with PHD function. Thus, several metals are known to induce HIF activity probably by direct replacement of iron in the catalytic center of the enzymes or by disturbing hydroxylation by preventing the completion of the enzyme's redox cycle (Schofield and Ratcliffe 2004; Wenger *et al.* 2005; Ke and Costa 2006). Iron chelators (deferoxamine (DFX), cyclopirox olamine) are well-known for their stabilization of HIFα. Similar to the substitution of iron by other metals, the iron in the catalytic center of the hydroxylases is chelated, inactivating the enzymes and thereby stabilizing HIFα (Hirsila *et al.* 2005). Another possibility to block HIFα hydroxylation is the direct inhibition with designed inhibitors such as dimethyloxalylglycine, a 2-oxoglutarat mimicking drug (Wirthner *et al.* 2007). Other potent inhibitors of the PHDs are succinate (the product of the enzyme reaction itself) and the chemically related fumarate, both compounds of the citric acid cycle (Isaacs *et*

al. 2005; Pollard *et al.* 2005; Selak *et al.* 2005; King *et al.* 2006) (Schofield and Ratcliffe 2004).

1.2.2 Other means to modulate HIF activity

In addition to the mechanisms governing HIF activity described above, various other stimuli dictate the outcome of HIF target gene activation. Several growth factors (PDGF, FGF-2, IGF-2, etc.) and cytokines (e.g. TFG-1β, TNFα, IL-1β) as well as oncogenes (ras, src) or tumor suppressor mutations (PTEN, pVHL, FH, SDH) have been shown to up-regulate HIF activity (Karni *et al.* 2002; Stiehl *et al.* 2002; Wenger 2002; Bilton and Booker 2003; Dery *et al.* 2005; Gort *et al.* 2006). One way of HIFα regulation by these factors may originate through direct phosphorylation of HIFα and thereby modulating HIF activity. As a matter of fact, HIF is strongly phosphorylated, but HIFα degradation seems not to be affected by these posttranscriptional modifications pointing to a mechanism different from HIFα protein stabilization (Bilton and Booker 2003). Moreover, phosphorylation of threonine 796 was reported to prevent the inhibitory hydroxylation by FIH (Gradin *et al.* 2002), while MAPK pathway activation leads to increased transcriptional activity of HIF (Sang *et al.* 2003; Brahimi-Horn *et al.* 2005). However, the main impact of these factors on HIF regulation is through up-regulation of protein translation (Bilton and Booker 2003; Bardos and Ashcroft 2004). Enhanced translation appears to be sufficient to overcome degradation or to titer out the PHDs, resulting in detectable HIFα protein (activity?) even under normoxic conditions.

Basic levels of HIF-1α are subject to other control mechanisms, such as the multi-functional scaffold protein receptor for activated C-kinase 1 (RACK1) and the chaperone heat shock protein 90 (HSP90) (Liu and Semenza 2007). HSP90 has previously been recognized as HIF interactor and stabilizer (Katschinski *et al.* 2004). RACK1 in this scheme counteracts HIF-1α stabilization by HSP90 by competing for interaction with the PAS-A domain of HIF-1α. Upon binding to the PAS domain, RACK1 mediates the contact to the same ubiquitin-complex as pVHL, ultimately leading to HIF-1α polyubiquitination and degradation (Fig.5). This oxygen-independent HIF-1α degradation pathway could be stimulated in RCC4 cell lines (which are

pVHL deficient and therefore contain high endogenous HIFα levels) by the use of HSP90 inhibitors (Liu *et al.* 2007). The HSP90 inhibitors geldanamycin and 17-allylaminogeldanamycin (17-AAG) exert their action by binding and blocking the ATPase domain of the chaperone and thus abrogate the stabilizing interaction of HSP90 with its client proteins, in this case HIF-1α.

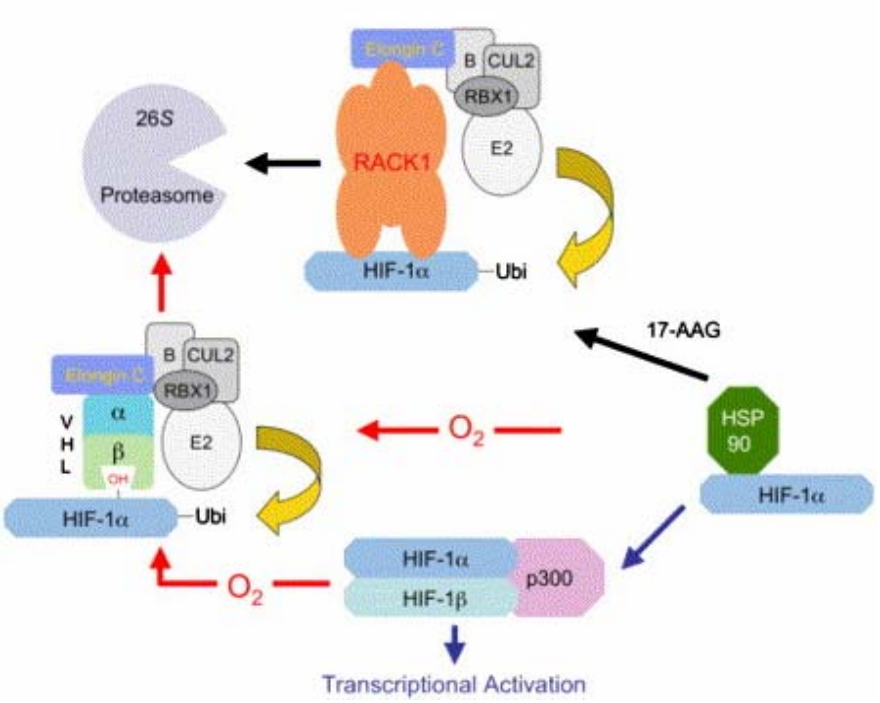

Figure 4. Degradation of HIF-1α by the 26S proteasome. RACK1 binding to the transcription factor is independent of hydroxylation. RACK1 (analog to pVHL) mediates the interaction to the ubiqutin ligation complex. HSP90 competes with RACK1 for binding to HIF-1α. 17-AAG abrogates HSP90 interaction. This results in enhanced RACK1 dependent degradation of HIF-1α according to Liu (figure taken from Liu and Semenza 2007)

1.3 HIF and tumor growth

Considering the different ways of HIF regulation, it is not surprising that the HIFα subunit is detectable in the majority of human tumors in contrary to normal tissue of the same origin (Aebersold *et al.* 2001; Maxwell 2005).

The enhanced HIFα levels in malignant tissues are accompanied by elevated expression levels of HIF-target genes (Wenger *et al.* 2005). These genes are central to the regulation of cellular metabolism, nutrition control, angiogenesis and pH (Semenza 2003). Thus, HIF helps to meet the high nutrition demand of highly proliferative tumor cells and contributes to equilibrate the cellular metabolism.

Clearly, HIF is at the heart of the regulatory systems controlling neovascularization (Pouyssegur *et al.* 2006). Vasculogenesis is primarily governed by oxygen supply and HIF activation leads to the expression of several angiogenic factors, among them the key regulator vascular endothelial growth factor (VEGF) (Vogelstein and Kinzler 2004). Moreover, the requirement of HIF for neovascularization and tumor growth has been shown Tang. (Tang *et al.* 2004).

Besides its participation in the formation of new blood vessels, the uptake of nutrients (glucose, iron) is enhanced by HIF and glycolysis is up-regulated, whereas oxidative metabolism is reduced (Wenger 2002; Papandreou *et al.* 2006). This leads to anaerobic ATP generation (Pasteur effect) and accumulation of lactate (Seagroves *et al.* 2001) and might explain the increased glycolysis by cancerous tissue when compared to their healthy counterparts; a phenomenon first described by O.H. Warburg. Lactate is transported by the H^+/lactate monocarboxylate transporter (MCT) into the extra-cellular milieu and contributes to acidosis (Pouyssegur *et al.* 2006).

Acidosis is another feature of solid tumors apart from angiogenesis or the Warburg effect. Cellular pH-control is governed by Na^+/H^+ exchanger (NHE) and NHE1 is HIF-dependently induced (Shimoda *et al.* 2006).(Cardone *et al.* 2005). However, HIF dependent expression of membrane-bound, extra-cellular carbonic anhydrase IX (CAIX) drives the alkalinization of the intracellular milieu but enforces the acidification of the extra-cellular milieu. CAIX converts water and carbon dioxide (formed during anaerobic glycolysis) to bicarbonate and protons, thereby up-regulates the extra-cellular pH. Bicarbonate, a weak base, buffers the intra-cellular milieu following transport into the cell by the sodium-bicarbonate co-transporter (NBC) or the anion exchanger (AE). Thus, HIF contributes to tumor acidosis and affects cellular pH-control (Pouyssegur *et al.* 2006).

In summary, HIF activity might explain some of the characteristics of malignant tissues. In general, HIF contributes to tumor growth and drives the surrounding tissue to (re)-establish conditions suitable for cellular life.

1.3.1 HIF and malignant progression

HIFs' support of tumor development (and thus malignancy) is based largely on their ability to mitigate angiogenesis and nutrition supply (Pouyssegur *et al.* 2006). However, during cancer progression other components of the HIF response may account for a more malignant state. Cell type specific HIF-dependent effects on apoptosis or metabolism contribute to HIF-dependent malignant progression as well.

The pathophysiological induction of HIF, as observed in neoplastic lesions, reflects the whole spectrum of HIF-target genes. (Wenger 2002; Greijer and van der Wall 2004). HIF target genes can be grouped in genes required for metabolism (glycolysis, mitochondrial respiration), nutrient supply (glucose uptake, iron uptake, angiogenesis, erythropoiesis), cellular survival, apoptosis, proliferation, cytoskeletal structure, mobility etc. (Semenza 2003; Wenger *et al.* 2005). Many of these genes support cellular survival under harsh conditions. In short, HIF comes into action to sustain cell survival and tissue homeostasis. Intriguingly, under pathophysiological circumstances HIF helps neoplastic alterations to endure, giving time for clonal expansion of progressively more malignant cells.

1.4 Tumors

1.4.1 Tumor definition

The term "tumor" originates from the Latin word *tumor* and is translated by "swelling". Tumor characterizes every abnormal growth of tissue including masses due to inflammation or malformations as well as extrusions of tissues due to rigidifications (e.g. ingrown splinter). This definition includes tissues spawn by dysregulated cell proliferation known as neoplasia i.e. new types of tissue. Neoplasia or cancer is subdivided into benign or malignant according to their ability to invade the underlying tissue. Further histopathology classification enables cancer researcher to group the majority of human neoplasia into tumors of the ephitelial tissue, termed carcinomas; into tumors originating from the mesoderm of the embryo, termed sarcomas; into cancer derived from hematopoietic tissues (e.g. lymphomas, leukemia); and into tumors arising from cells with connection to the nervous system (sometimes

also referred to as neuroectodermal tumors e.g. glioblastoma, retinoblastoma) (Weinberg 2007).

1.4.2 Tumor progression

The recognition of tumors cells as being descendants of normal tissue cells hints to a malfunction of the progeny (Bickers and Lowy 1989). Hanahan and Weinberg concluded "...cancer to be a disease involving dynamic changes in the genome" and postulate the hallmarks of cancer as a multi-step process of genetic alterations which leads from normal human cells to malignant cancer cells (Preston-Martin *et al.* 1990; Hanahan and Weinberg 2000).

In order to evolve into cancerous tissue, normal human cells have to circumvent several anti-cancer barriers that ensure the integrity of normal human tissue (Bartkova *et al.* 2005; Gorgoulis *et al.* 2005).

Taken together, this signifies a halt for a proliferating tumor mass until the required abilities were achieved by a clone and therefore points to a clonal expansion in a Darwinian way. (Fig. 1A)

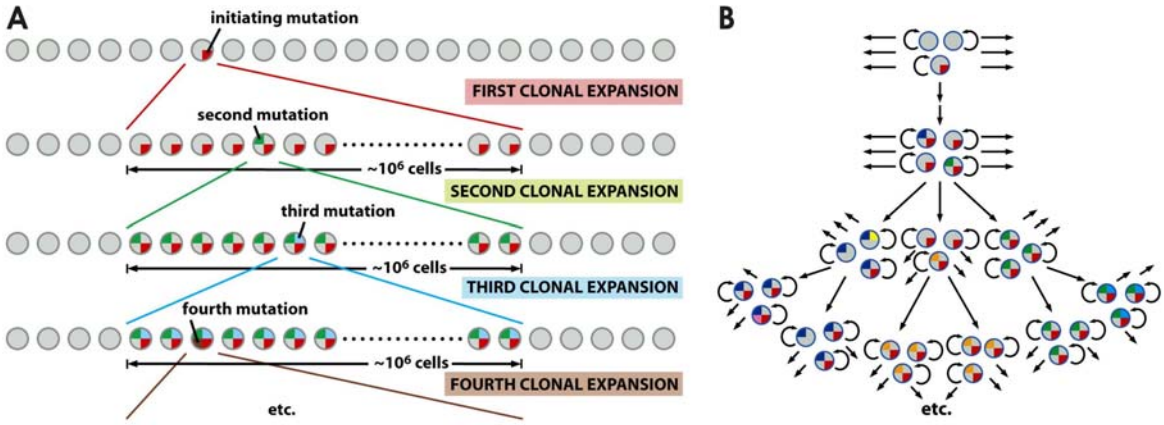

Figure 5. (A) Expansion in a Darwinian way requires selection for the fittest cell followed by expansion of the population. Further clonal expansion after a beneficial mutation eliminates less adapted clones from the population. (B) Cancerous progression viewed as heterozygote clonal evolution (figure taken from Weinberg 2007).

However the discovery of cancer stem cells, the contribution of epigenetic factors to malignant transformation and the possible rapid rate of de-differentiation of cancer cells requires adapting this simplified classical perception of cancer cell selection to a contemporary view of cancer evolution

(Grady and Markowitz 2002; Lobo *et al.* 2007) (Fig. 1B).The road to a fully developed malignant state might be different for any kind of human tissue but on the molecular level similar events have to take place to pass the barriers imposed by the metazoan evolution to allow for cancer progression (Lane 2005; Weinberg 2007).These anti-cancer barriers were depicted as aberrant mitogenic signaling, loss of cell cycle control, avoidance of apoptosis, immortalization and the ability to trigger the angiogenic switch (Hanahan and Weinberg 2000).

1.4.2.1 Aberrant mitogenic signalling

For a human cell to proliferate, external growth stimuli are needed which are converted by transcriptional and translational induction into gain of mass and division cycles. The conversion of these stimuli into the corresponding cellular response is best characterized by the case of the RTK-ras-MAPK pathway (Chang and Karin 2001; Alberts 2002). Thus, every cell makes use of several distinct mitogenic signaling pathways that combine and control the adequate proliferation of each cell. However, every malfunction of each step of a given pathway has the potential to induce aberrant mitogenic signaling (Porter and Vaillancourt 1998) (Hunter 1997).

1.4.2.2 Loss of cell cycle control

During metazoan development, single cells had to learn to communicate together to ensure that a cell body will emerge out off a single cell (Lane 2005). This communication yielded into a cell cycle clock which strictly depends on external permission signals to proceed (Tapon *et al.* 2001). Additionally, external anti-growth stimuli (e.g.TFG-β) are applied to keep or to bring a cell into a quiescent state. For a cancer cell to avoid this status means to neglect the superimposed cell cycle state (Blagosklonny and Pardee 2002). The cell cycle machinery is tightly controlled, but in a simplified view all signals finally converge in the phosphorylation status of pRB (and its cousins p107, p130, collectively called pocket proteins) (Weinberg 2007). pRB on the other hand controls the R point transition and only hyperphosphorylated and inactivated pocket proteins will lead to the passage of the G1 phase of the cell

cycle (Bartek *et al.* 1997) (Fig.2). Thus, de-regulated cell cycle control, vastly influenced by pRB, inexorably provokes cancer progression.

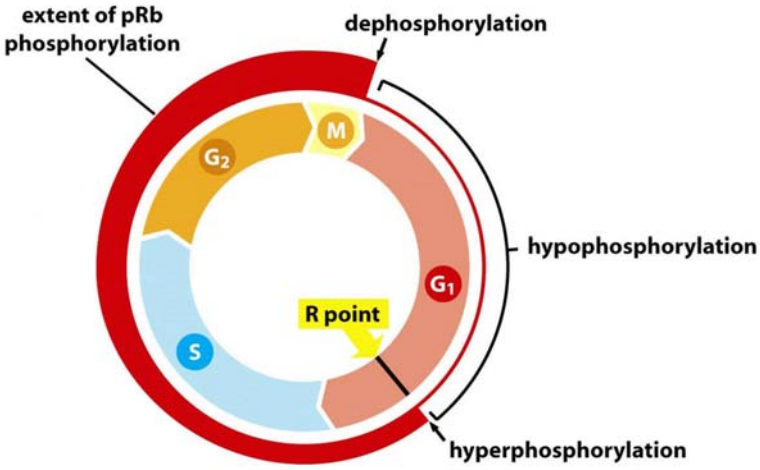

Figure 6. Passage over the restriction point reflects pRB phosphorylation state. The cell cycle will be completed after R point transition regardless of exogenous signaling. Phosphorylation of the pocket protein is performed by cell cyclin dependent kinases initiated by mitogenic stimuli (figure modified from Weinberg 2007).

1.4.2.3 Avoidance of apoptosis

The master guardian over the cell state p53 is mutated or inactivated in most (if not all human cancers) (Weinberg 2007). This high rate of affected p53 in cancer progression underscores the prominent role of this protein in keeping tissue homeostasis in order. Upon inadequate stimuli such as: over-shooting proliferation signaling, loss of adherence to the extracellular matrix or damaged DNA, p53 is stabilized and thereafter induces cell cycle arrest, and by up-regulation of proapoptotic factors (e.g. bax, puma, noxa), shifts the balance of the cell fate towards apoptosis (Levine 1997; Giaccia and Kastan 1998). In order to progress to a malignant state a precancerous cell therefore has to shift the balance of pro- and anti-apoptotic factors. As a result of the complex interactions, which determine the onset of apoptosis, and the abundant factors and signal pathways involved, avoidance of apoptosis seems most promising by targeting a key regulator such as p53 - but of course every factor influencing the execution of the death program or every contribution to one side of the balance of pro- vs. anti-apoptotic factors might

prohibit a cell to correctly engage into the cell death program (Green and Evan 2002; Igney and Krammer 2002).

1.4.2.4 Immortalization

Mammalian cells rely on a specific number of cell divisions after which they remain insensitive towards division stimuli but keep metabolic active. This process is termed replicative senescence and was traced back to an accumulation of DNA damage in the cell (Cristofalo *et al.* 2004). The cells thus will stop proliferating once the burden of DNA damage reaches a certain threshold. Apart from repeated replication rounds, other stresses are known to induce an indistinguishable cellular state. Among this stresses are DNA damage, aberrant mitogenic signaling (e.g. ras), oxidation or xenobiotic exposure (Weinberg 2007). Circumvention of senescence is made possible for rodent cells by inactivating key regulators (pRB, p53) by transfection with large T-antigen leading to immortalization. This state of unlimited replicative ability is also observed by spontaneous immortalization in cultured rodent fibroblasts. In this case, however, natural selection for inactivated key regulators results in immortalization (Katakura *et al.* 1998).

Human cell lines, however, will not become immortalized as it is the case for cells with rodent origin but will result in a state called crisis, accompanied by heavy apoptosis after an additional number of cell doublings beyond the usual entrance into senescence (Weinberg 2007). Characteristic for cells in crisis is the appearance of fussed chromosomes and eroded telomeres. The molecular origin of the appearance of this phenotype is coupled to human telemorase regulation. Expression of the catalytic subunit of the enzyme hTERT is repressed in humans postembryonic cell lineages (in contrary to mice), resulting in telomerase independent telomere maintenance. Hence, telomeres in human cells shorten after each replication step until they are no longer able to protect the ends of the chromosomes (Stewart and Weinberg 2006).

1.4.2.5 The angiogenic switch

The more a tumor gains mass the more distant from the vasculature some cells become. At some point in the growth of a tumor, support of nutrition (and

disposal of cell waste) will be restricted for those cells. This leads to a proliferation stop and slows down tumor progression. Large necrotic areas will form (Kaanders *et al.* 2002; Naumov *et al.* 2006). The sudden emergence of new capillaries inbetween the neoplastic tissue restores the cells with proliferative energy and gives rise to further tumor progression. For a healthy tissue, neoangiogenesis is restricted and cancer tissue has to pass this barrier in order to evolve further (Hanahan and Folkman 1996; Bergers and Benjamin 2003). The onset of this process of increasing angiogenesis, "the angiogenic switch", appears as the first milestone towards a highly vascularized tumor, paralleled by poor clinical outcomes (Heimann and Hellman 1998; Hlatky *et al.* 2002; Weinberg 2007). To build up a dense network of new vessels, the tumor relies on macrophage and fibroblast recruitment and the subsequent infiltration of endothelial precursor cells attracted by the same cytokines (PDGF, FGF, VEGF, TGF-β etc.) as released by damaged vasculature (Bingle *et al.* 2002; Knowles and Harris 2007).

The dramatic changes observed in neoplastic tissue, leading to new capillaries, are similar to the mechanisms observed during wound healing (Dvorak 1986; Singer and Clark 1999; Weinberg 2007). This ensures oxygen availability and cell survival for a cancerous mass since more distant areas from blood vessels experience an increasingly hypoxic environment (Brown and Giaccia 1998; Hockel and Vaupel 2001; Naumov *et al.* 2006). Paradoxically, tumors with large hypoxic regions are prone to develop an even more aggressive phenotype (Vaupel and Mayer 2007; Weinberg 2007). This might be explained by the function of the hypoxia-inducible factor (HIF) that up-regulates, amongst others, genes involved in neoangiogenesis. The protein levels of HIF strongly correlate with poor clinical prediction and tumor therapy resistance.(Wenger 2002; Linden *et al.* 2003; Tang *et al.* 2004; Manalo *et al.* 2005; Moeller and Dewhirst 2006; Pouyssegur *et al.* 2006).

1.5 Cancer therapy

The gold standard for cancer therapy is based on the three conventional methods: surgery, chemotherapy and radiotherapy (Lodish 2000; Stenner-Liewen *et al.* 2006). Due to the progress in the development of these methods

and due to the increased sensitivity of early diagnosis, cancer therapy nowadays is able to cure a range of occurring malignant transformations (Alberts 2002). However, depending on localization and origin, some tumors are difficult to treat, resulting in a high rate of mortality and an increase of therapeutic side-effects. Modern clinical approaches try to lower treatment burden and enhance therapeutic efficiency by complementing classical therapies (Alberts 2002). The goal of these attempts towards more specific cures is to discover characteristic attributes of transformed tissue. These unique traits serve as molecular targets to directly induce tumor regression or render cancerous cells more sensitive to therapy (Balis 2002; Stenner-Liewen *et al.* 2006). Therefore, the prerequisite to designed drugs is a fundamental understanding of the molecular processes in the cancerous lesions as well as in healthy tissues. Unfortunately, molecular action of the conventional remedies remains only partially resolved and modern approaches, besides success in a few rare cases, are rather ineffective (Weinberg 2007).

In general, traditional nonsurgical strategies to eliminate cancer cells induce cell death by activation of the apoptosis program in one or the other way but do not specifically target malignant cells (Fisher 1994; Lowe and Lin 2000; Johnstone *et al.* 2002). Some degree of selection is achieved by applying treatments that preferentially affect (fast) proliferating cells. One way is to disrupt DNA to such extend as to overrule repair. Proliferation of cells with damaged DNA is abrogated leading to cell cycle stop and apoptosis (Lodish 2000; Alberts 2002)

1.5.1 Etoposide

Etoposide is one of the most important drugs in cancer chemotherapy and is used to treat different type of cancers, including testicular, ovarian, lung and stomach cancer. In the clinics, etoposide is intravenously applied. Side-effects include lowered resistance to infection due to reduced white blood cell production, anemia due to reduced red blood cell production, bleeding due to reduced platelet production and hair loss. Chemically, the compound is derived from podophyllotoxin a substance isolated from the American mayapple tree (*podophyllum peltatum*). The semi-synthetically modified toxin is also known as eposin, vepeside or VP-16. Etoposide exerts its action by

inhibition of topoisomerase II, an enzyme used to unwind curled DNA-strands. Topoisomerase II is needed for replication of the DNA as well as for transcription to RNA by the respective polymerases. In order to unwind the DNA strand, the enzyme cuts one side of the DNA double-strand and passes the other strand through the newly formed gap before relegation. The etoposide inhibited isomerase yields a DNA-topoisomerase intermediate that collapses by a polymerase complex, leading to DNA double-strand breaks. Therefore, the cellular concentration of topoisomerase II is critical to the toxicity of the drug and the more topoispomerases II, as observed in proliferating cells (of a neoplastic tissue), the more detrimental the drug (Meresse *et al.* 2004).

1.5.2 Hypoxia, HIF and therapy resistance

During solid tumor growth, hypoxic to anoxic areas are commonly encountered owing to inadequate vasculature of the growing malignant transformation and the resulting imbalance in oxygen supply and demand (Brown and Giaccia 1998). Thus oxygen-deprived cells will become apoptotic and necrotic regions will form in the tumor, as well (Kaanders *et al.* 2002). Therapy outcome of tumors with alternating areas of hypoxia and normoxia is difficult to predict and hypoxic regions correlate with therapy resistance (Hockel and Vaupel 2001; Vaupel and Mayer 2007).

Hypoxic therapy resistance is a well-established aspect in solid tumor treatment, however, the underlying mechanisms are not completely understood. When it comes to HIF function, as master controller of the hypoxic response, things are even more uncertain.

Based on HIF function in vascularization it is evident that this transcription factor is a strong, tumor-growth promoting element. Other important aspects of HIF function, regulation of cellular metabolism (glycolysis, oxidative phosphorylation) and its involvement in apoptosis seem to contribute to the tumor promoting action of HIF but still need clarification (Pouyssegur *et al.* 2006). The very same reasons that suggest HIF as a tumor promoter are discussed as reasonable arguments for HIF's role in cancer therapy resistance (Moeller and Dewhirst 2006).

HIF might impact on therapy resistance by mediating vascularization and ensuring tumor growth by stimulation of endothelial cell survival. This, however, does not explain the cellular effects of HIF on therapy resistance observed in cell culture studies (Zhang *et al.* 2004; Williams *et al.* 2005; Brown *et al.* 2006; Hussein *et al.* 2006; Song *et al.* 2006). According to these studies HIF is a negative factor in tumor therapy, possibly acting as "survival factor". Indeed, some authors try to establish an effect of HIF on apoptosis, leading to enhanced resistance through a shift from pro- to anti-apoptotic factors (BID, BAX) (Erler *et al.* 2004). However, contradictory reports question the findings (Biju *et al.* 2004). Moreover, the use of anoxic conditions, that induce p53 and thereby a presumable effect of p53, diminish the importance of these observations (Schmid *et al.* 2004). The exact role of HIF in induction of apoptosis is not clear and might even depend on the oxygen partial pressure or glucose abundance (Greijer and van der Wall 2004; Kilic *et al.* 2007).

Another approach to explain HIF's role in cancer therapy resistance was to circumvent the possible implication of p53 in apoptosis by the use of p53 inactivated cell lines. By comparison of two p53 inactivated cell lines, one wild-type and the other deficient for HIFα, a difference in the sensitivity towards DNA double-strand break-inducing drugs could be observed (Unruh *et al.* 2003). Surprisingly other compounds did not provoke the same differential cell kill. Together with a plasmid rejoining assay this data led to the assumption that factors involved in the maintenance of DNA, more precisely in the re-ligation of DNA double-strand breaks, may depend on HIF for proper action.

1.6 DNA-repair

The construction plan of every single cell in our body is located in the nucleus itself. Wrapped around proteins, tangled together and fixed to a scaffold, but altogether nicely protected, the DNA serves as encoded sketch for its own duplication (Richmond 2006). In this way DNA templates are passed from one generation to another. Shelter from possible harm is one mean to assure the integrity of the genome. In spite of this protecting architecture, DNA is

subjected to chemical and physical damage (Weinberg 2007). Therefore, without surveillance and restoration the code would soon be mutated to such an extent as to abrogate genome duplication or to enhance the rate of tumor incidence and progression. Thus, a cell has developed “care keeper” enzymes to survey and fix DNA (Scharer 2003).

1.6.1 Double-strand breaks

DNA double-strand breaks (DSBs) are one of the many DNA damages a cell has to suffer from and is believed to be one of the most severe types of lesions (Valerie and Povirk 2003). DSBs arise from exogenous agents such as ionizing radiation or radio-mimetic compounds (chemotherapeutic agents: etoposide, cisplatin). DSBs are also formed by reactive oxygen species (ROS) generated by endogenous sources (peroxisomal, mitochondrial) (Pierce *et al.* 2001). Furthermore DSBs are caused by mechanical stress on chromosomes or by collapse of replication forks when the replication machinery encounters a DNA single-strand break or other DNA distortions and by programmed cleavage during various biological events such as V(D)J recombination during immunoglobulin gene rearrangement or meiotic recombination or in the course of an inflammatory response. DSBs lead to cell cycle arrest and, if unrepaired, to cell death (Collis *et al.* 2005). Errors during repair may lead to insertion or deletion mutations or to genomic instability through chromosomal translocation. The DSB response activates the repair machinery and transiently arrests cell cycle progression which involves distinct enzymes, many of which assemble at the damaged site (Jackson 2002; Valerie and Povirk 2003).

1.6.2 Histone H2AX

One of the earliest events in the DSB response is the rapid and massive phosphorylation of H2AX a histone variant of H2A (Olive and Banath 2004) The isoform H2AX accounts for 2%-25% of the total histone H2A of a cell and is ubiquitously distributed throughout the genome (Rogakou *et al.* 1998). A few minutes after DSB insult and several hundred of kilobases around the break, phosphorylation of serine 139 on the carboxy-terminal end of the histone takes place (Rogakou *et al.* 1999; Pilch *et al.* 2003; Tsukuda *et al.*

2005). Phosphorylated H2AX is termed γH2AX. Immunohistochemical detection of γH2AX reveals the formation of visible foci proportional to the irradiation dose and overlaps with break sites (Limoli and Ward 1993; Rogakou *et al.* 1998; Rogakou *et al.* 1999; Banath and Olive 2003; Banath *et al.* 2004). The role of γ-H2AX in DSB repair is not entirely understood but is necessary for sustained recruitment of repair factors and to restructure chromatin (Celeste *et al.* 2003; Chowdhury *et al.* 2005; Franco *et al.* 2006). H2AX knock-out mice are viable but inonizing radiation (IR) sensitive, immunocompromised and suffer from increased genomic instability, underscoring the involvement in DNA repair. DNA damage-induced phosphorylation is attributed to at least three members of the PIKK (phosphatidyl inositol kinase related kinase) group of kinases DNA-activated protein kinase (DNA-PK), Ataxia-telangiectasia mutated (ATM), ATM- and rad3-related (ATR) (Pilch *et al.* 2003; Fernandez-Capetillo *et al.* 2004; Kurose *et al.* 2005; Reitsema *et al.* 2005).

1.6.3 The phosphatidyl inositol 3-kinase related kinases (PIKK)

The PIKKs form a family of six members (ATM, ATR, DNA-PK, mammalian target of rapamycin (mTOR), suppressor of morphogenesis in genitalia-1 (hSMG-1), transformation/transcription domain-associated protein (TRRAP)) of high molecular weight proteins. Five of them bear serine-threonine kinase activity, whereas TRRAP has a catalytic domain, although without phosphotransferase activity. The element that groups the enzymes into the large superfamily of the PIKKs is the catalytic domain with a high similarity in the DNA sequence, although the phosphorylation of proteins and not lipids is favored. Characteristic for the family of PIKKs, apart of the catalytic domain, are two domains termed FAT (**F**RAT, **A**TM, **T**RAPP) and FATC **(F**RAT, **A**TM, **T**RAPP, **C**-terminal) which flank the catalytic core and which are necessary for the catalytic efficiency of the enzymes, and finally a N-terminally located domain of α-helical repeat subunits called HEAT (**h**untingtin, **e**longation factor 3, **a** subunit of protein phosphatase 2A and **T**OR1) presumably used for protein-protein interaction (Abraham 2004).

1.6.3.1 ATM- and rad3-related (ATR)

ATR is an essential member of the PIKK family since homozygous mutation of the ATR locus in mice not only leads to embryonic lethality, but even cells could not be cultivated (Bakkenist and Kastan 2004). Therefore, ATR is needed for the survival of a cell and the organism, hinting to roles in development and/or cell cycle progression. Established is the requirement of ATR for appropriate signaling after DNA damage in course of replication or after disruption of proceeding replication events (Shechter *et al.* 2004). ATR seems to be activated by various DNA-damaging agents, replication inhibitor, UV irradiation, single-stranded DNA (ssDNA) gaps, severe hypoxia, and during ATMs response to DNA DSB (Yang *et al.* 2003; Bakkenist and Kastan 2004). Apparently, ATR is activated by replication protein A (RPA) covered ssDNA (Shechter *et al.* 2004). ATR interacting protein (ATRIP) binds RPA-ssDNA intermediates and thereby recruits ATR (Falck *et al.* 2005). Activated ATR will slow down or halt cell cycle progression by targeting several proteins implicated in cell cycle control (Shechter *et al.* 2004).

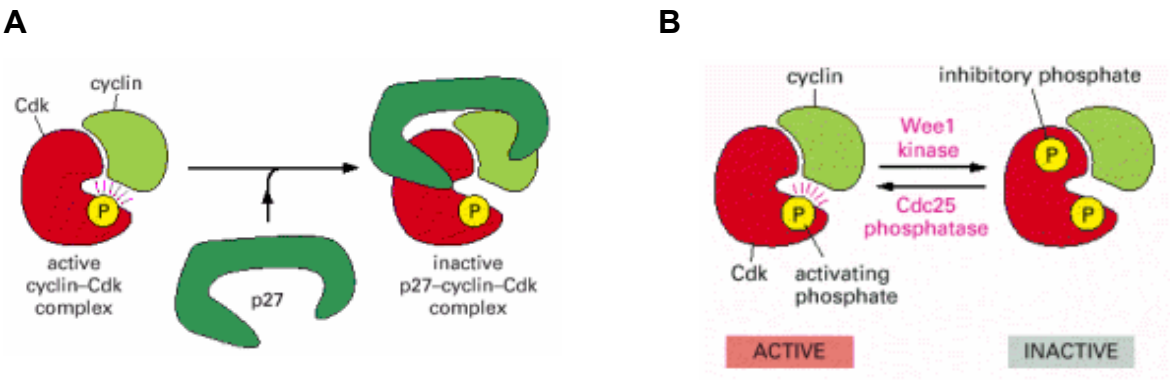

Figure 7. Inactivation of active cyclin-CdK dimers (A) CDKI inhibitor binds to the complex and keeps it inactive, here shown for p27 (a cousin to p21) (B) Activation of the kinase complex is possible by removing a phosphate group. The activating phosphatase is called cdc25. Degradation of cdc25 results in inhibited cyclin-Cdk dimers and cell cycle arrest (figure taken from Alberts 2002).

Cell-cycle progression depends largely on the activity of dimeric complexes with kinase activity. Cell cycle halt signifies to inactivate these complexes or to keep these complexes in an inactive state. One possibility is to physically block the kinase, like the cyclin-dependent kinase inhibitors (CDKI) do, by complexing with the cyclin-Cdk dimer (Weinberg 2007) (Fig.6A). Other means

to kinase inactivation include allosteric interaction or modification. Indeed control over S-phase transition is achieved by the interplay of inactivating phosphorylation of the Cdk complexes and activating removal of the phosphate group by a phosphatase (cdc25) (Alberts 2002) (Fig.6B). ATR triggers cell cycle arrest by phosphorylation of p53 which in turn up-regulates $p21^{waf1/cip}$, a CDKI, and by activation of Chk1 a cell cycle regulatory kinase resulting in phophorylation and degradation of cdc25 (Chen and Sanchez 2004; Shechter *et al.* 2004).

1.6.3.2 Ataxia-telangiectasia mutated (ATM)

ATM is the key transducer of the DNA DSB response. In undamaged cells ATM resides as inactive dimer. Upon insult, dimer dissociation and autophosphorylation on serine 1981 result in activation of the large kinase and subsequent phosphorylation of ATM target proteins in the surrounding of the break (Bakkenist and Kastan 2004). Active ATM is recruited to DSB by Nijmegen breakage syndrome 1 (Nbs1), a part of the evolutionarily conserved Mre11, Rad50, Nbs1 (MRN) complex (Falck *et al.* 2005; Lee and Paull 2005). Autophosphorylation is defective in Nbs1 or Mre11 compromised cells. Thus, MRN is needed for the initial activation and amplification of the ATM signal. In addition, Nijmegen breakage syndrome, A-T like Disorder (ATLD), caused by mutation in the MRE11 gene, and ataxia-telangiectasia share common clinical manifestations and overlapping cellular characteristic (Paull and Lee 2005). AT patients are deficent for ATM function and exhibit, amongst others, a pronounced radiosensitivity, increased risk of cancer, premature aging and immune deficiencies. Cells from AT patients do not respond appropriately to ionizing radiation, as reflected by enhanced cell death, enhanced chromosomal breakage and defective cell cycle checkpoints (Bakkenist and Kastan 2004). Similar to its cousin ATR, cell-cycle control is obtained by phosphorylation of p53 and inactivation of cdc25. However Chk2 rather then Chk1 is the mediator in the ATM response to DSB (Ahn *et al.* 2004; Kurz and Lees-Miller 2004).

1.6.3.3 DNA depended protein kinase (DNA-PK)

The function of the third PIKK involved in DSB repair clearly deviates from the mediator kinases ATM or ATR that organize the DSB-response. DNA-PK, however, is directly implicated in the repair event as part of the end-fixing and relegation machinery (Weterings and van Gent 2004). Even though p53 phosphorylation by DNA-PK may be observed in vivo and thus contributes to cell cycle regulation and apoptosis, the main task remains in soiling two DNA ends together (Collis *et al.* 2005). When DNA breaks, the large kinase complex is located on the DNA ends, acting as a scaffold for other repair proteins. The repair event is additionally directed by the kinase through the addition of phosphate groups to several key proteins. End-ligation presumably is supported by directly promoting pairing of the broken ends (Spagnolo *et al.* 2006). DNA-PK consists of three components: the heterodimeric Ku complex with its two subunits of approximately 70 and 80 kDa and the catalytic part called DNA-PKcs (catalytic subunit) (Spagnolo *et al.* 2006). The Ku complex has been originally discovered as autoantigen ("Ku" is derived from the first two letters of the original patient`s name) (Downs and Jackson 2004). The Ku complex is conserved in eukaryotes and the core complex of the dimer was discovered in archaea as well as bacteria (Doherty and Jackson 2001). The cellular localization of the dimer appears restricted primarily to the nucleus, both subunits having a nuclear localization signal (NLS). However, cytosolic Ku70 has been attributed a bax binding ability, exerting a possible anti-apoptotic function, as well (Koike *et al.* 2001; Sawada *et al.* 2003; Downs and Jackson 2004). Nevertheless, the principal form detected in the cell is the stable Ku70-Ku80 dimer, indicating a stabilizing effect off the subunits upon complex formation (Downs and Jackson 2004). The observed interdependence is further underscored by Ku80 deficient cell lines, possessing only little Ku70 (and vice versa) as well as by the reconstitution of a Ku80-lacking phenotype with Ku80 that leads to the restoration of both (Downs and Jackson 2004). The homology between the two proteins hints to a common ancestor. The major difference is found in the C-terminal part. Whereas Ku70 possesses a SAP domain with low DNA binding affinity, Ku80 has a DNA-PKcs binding-site (Downs and Jackson 2004). Three dimensional

analyses revealed a ring-like structure for the Ku complex which may encircle DNA ends without any further sequence specificity - but with Ku70 aligned distal to the end when compared to Ku80 (Walker *et al.* 2001). Recruitment of the large catalytic subunit by the Ku-complex to free DNA ends then follows, since the trimeric complex requires the presence of free DNA ends for strong and stable interaction (Weterings and van Gent 2004). Association of the catalytic subunit with the Ku complex on DNA stimulates the activity of the kinase and leads to phosphorylation of downstream targets – eventually the Ku ring winds interior, placing the DNA-PKcs at the edge of the DNA strand, thereby protecting the ends from nucleases or physical damage (Meek *et al.* 2004). Two DNA-PKcs proteins on two opposite ends then contact each other, possibly yielding a synaptic complex which is a prerequisite for ligation (Spagnolo *et al.* 2006). In contrary to the Ku complex DNA-PKcs is only found in vertebrates. Thus, it has been speculated that other homologous of PIKKs exist in lower eukaryotes or that functions of DNA-PKcs are specific to higher organisms e.g. repair-dependent chromatin remodeling (Khanna and Jackson 2001; Jackson 2002; Lees-Miller and Meek 2003). Mice deficient for DNA-PKcs have defective immune systems caused by a disturbed B- an T-cell maturation due to its role in V(D)J-rejoining. These mice are not only comprised in their immune system but are also hypersensitive to IR (Lees-Miller and Meek 2003). Both events require the ligation of two opposed DNA ends. Owing to the central role of DNA-PKcs in religation of DNA ends, this process is abolished in the knock-out mouse, resulting in the observed phenotype (Lieber *et al.* 2004). The phenotype of Ku-deficent mice is similar to DNA-PKcs mutants but additionally marked by reduced body weight at birth, slow growth and shorter life expectancy. Therefore, additional functions of the Ku heterodimer e.g. in telomere maintenance are expected (Lees-Miller and Meek 2003; Downs and Jackson 2004).

1.6.4 Homologous recombination repair *versus* non-Homologous end joining

A vertebrate cell relies on two major pathways when it comes to fixation of DNA DSB: HR or NHEJ (Pierce *et al.* 2001).

Even though both pathways lead to fixed DNA ends, the two mechanisms are different. Whereas HR relies on a template to keep the original sequence preserved in an error-free process, NHEJ religates broken DNA ends without sequence consideration and therefore is per se an error-prone process (Lisby and Rothstein 2005). Due to the dependence on a template for action, HR is restricted to the late S/G2 phase of the cell cycle when sister chromatids are aligned and recombination is possible. On the other hand, NHEJ ligates broken DNA ends as the major mechanism during G0/G1 phase of the cell cycle but still persists in the later cell cycle phases (Lees-Miller and Meek 2003; Lieber *et al.* 2004).

1.6.4.1 Homologous recombination repair (HR)

HR is performed by a multitude of proteins including RPA, Rad51, Rad52, Rad54, MRN, BRCA1 and BRCA2 which are directly involved in the repair event or direct the repair event by acting as scaffold proteins.

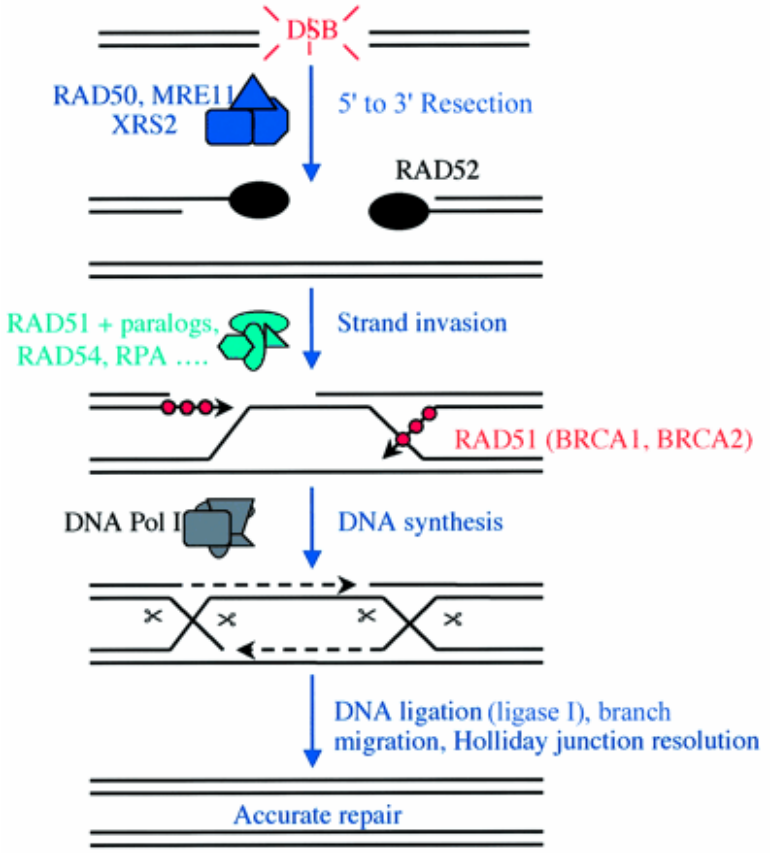

Figure 8. Homologous recombination repair. After strand alignment of the broken strand with the corresponding template, HR starts with nucleolytic 5`to 3` resection of the strand. Rad51 assists strand invasion and homologous pairing in concert with numerous additional proteins leading to a recombination intermediate. After DNA synthesis the restored strands are re-igated. Final resolution of the recombination intermediate by cleavage and ligation results in two intact DNA double helices (figure taken from Jackson 2002).

Mediator proteins which promote the repair event are ATM and ATR. In short, after DSB recognition the helix is cut partially, leading to single-stranded DNA coated by RPA protein. This will subsequently be complexed by Rad51 which is necessary for strand invasion and exchange. Further unwinding of the double-strand is assisted by the Rad51 homologous and Rad52 as well as by Rad54 and helicases such as WRN or BLM helicase, leading to a recombination intermediate. Finally, strand replication by a polymerase and ligation of the resulting gap restores the DNA strand and completes repair (Jackson 2002) (Fig.7).

1.6.4.2 Non-homologous end joining

The mediator kinase ATM and the MRN complex have a function in the early steps of DSB repair by NHEJ, probably in signaling the break to the cell and in trimming the ends for subsequent repair. However, NHEJ does not strictly depend on these factors. The process is initiated by the Ku heterodimer binding to the ends of the break point. This leads to DNA-PKcs recruitment to the damaged site.

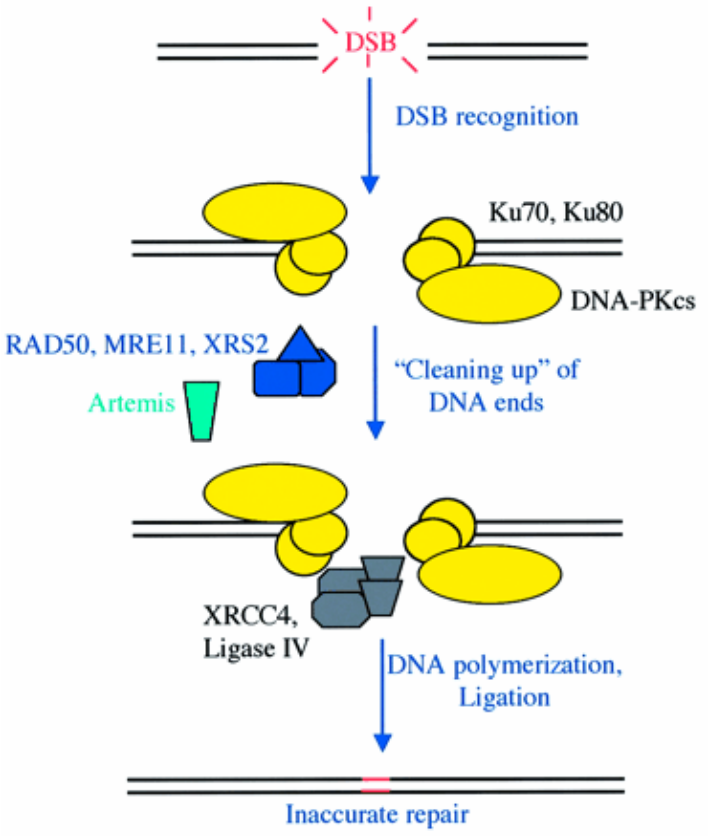

Figure 9. Non-homologous end joining. The broken DNA ends are immediately fixed and bound by the Ku-dimer. The Ku-DNA intermediate atractes and activates DNA-PKcs. The DNA-PK holoenzyme then physically bridges and stabilizes the break and phosphorylates other targets, probably to direct repair. Depending on the damage some broken ends need to be trimmed prior to ligation. The completed process results in a repaired DNA strand. However changes in the sequence are likely to occur. Thus, NHEJ is an error-prone process (figure taken from Jackson 2002).

In principal, the stabilized DNA ends could now be ligated by a ligase, but most often the DNA ends need to be processed first. Artemis, a nuclease is the most likely candidate for this function. After end resection and addition of a phosphate group by the terminal deoxynucleotidyl transferase (TdT), most probably polymerase μ fills up the existing gap and ligase IV in complex with XRCC4 (an adapter protein) connect the ends together (Jackson 2002) (Fig.8).

2 Results

2.1 Determination and modulation of prolyl-4-hydroxylase domain (PHD) oxygen sensor activity

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2.1.1 Abstract

The prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins hydroxylate hypoxia-inducible transcription factor (HIF) α-subunits, leading to their subsequent ubiquitinylation and degradation. Because oxygen is a necessary co-substrate, a reduction in oxygen availability (hypoxia) decreases PHD activity and subsequent HIFα hydroxylation. Non-hydroxylated HIFα cannot be bound by the ubiquitin ligase von Hippel-Lindau tumor suppressor protein and HIFα proteins thus become stabilized. HIFα then heterodimerizes with HIFβ to form the functionally active HIF transcription factor complex which targets approx. 200 genes involved in adaptation to hypoxia. The three HIFα prolyl-4-hydroxylases are clearly of different nature compared with the prototype collagen prolyl-4-hydroxylase which hydroxylates a mass protein rather than a rare transcription factor. Thus, novel assays had to be developed to express and purify functionally active PHDs and to measure PHD activity in vitro. There is also a need for such assays to functionally distinguish the three different PHDs in terms of substrate specificity and drug function. We provide a detailed description of the expression and purification of the PHDs, as well as of a HIFα-dependent and a HIFα-independent PHD assay.

2.1.2 Introduction

Cells sense changes in environmental oxygen availability by a group of enzymes which directly control the cellular response to low oxygen by destabilizing hypoxia-inducible factor (HIF) α subunits, the master transcriptional regulators of the hypoxic response. These oxygen sensing enzymes have alternatively been termed prolyl-4-hydroxylase domain (PHD), HIF prolyl hydroxylase (HPH) or egg laying defective nine homolog (EGLN). Three family members are known up to date: PHD1/HPH3/EGLN2, PHD2/HPH2/EGLN1 and PHD3/HPH1/EGLN3 (Bruick 2000; Epstein *et al.* 2001; Ivan *et al.* 2002). PHDs hydroxylate HIF-1α and HIF-2α at two distinct proline residues within the HIFα oxygen-dependent degradation (ODD) domain (Fig. 1A). Under normoxic conditions, prolyl-4-hydroxylation allows binding of the von Hippel-Lindau tumor suppressor protein (pVHL), leading to polyubiquitinylation and proteasomal destruction (Wenger 2002; Schofield and Ratcliffe 2004). Under hypoxic conditions, prolyl-4-hydroxylation is reduced, HIF-1α and HIF-2α become stabilized, heterodimerize with the constitutively expressed HIF-1β subunit aryl hydrocarbon receptor nuclear translocator (ARNT), and regulate the expression of a large number of effector genes involved in adaptation to low oxygen (Wenger *et al.* 2005). In addition, factor inhibiting HIF (FIH) hydroxylates an asparagine residue within the C-terminal transactivation domain. Oxygen-dependent asparagine hydroxylation blocks the recruitment of the CBP/p300 transcriptional co-activators and thereby regulates the transcriptional activity of HIFs (Hewitson *et al.* 2002) (Mahon *et al.* 2001; Lando *et al.* 2002)

PHDs do not represent static oxygen sensor molecules but rather are highly regulated themselves. Importantly, PHD2 and PHD3, but not PHD1, have been reported to be hypoxically induced at both the mRNA and protein levels (Epstein *et al.* 2001). Accordingly, elevated PHD2 and PHD3 levels have been demonstrated in a broad panel of established cancer cell lines (Appelhoff *et al.* 2004). Functional hypoxia response elements (HREs) are located in the promoter region of the human *PHD2* gene as well as in the first intron of the human *PHD3* gene, demonstrating that *PHD2* and *PHD3* are HIF

target genes themselves (Metzen *et al.* 2005; Pescador *et al.* 2005). Because the essential co-factor oxygen is basically lacking under hypoxic conditions, the HIF-dependent hypoxic increase in PHD abundance is somehow paradoxical and it has been suggested that PHD induction plays a role in accelerating the termination of the HIF response following re-oxygenation (Epstein *et al.* 2001; Appelhoff *et al.* 2004; Aprelikova *et al.* 2004; Marxsen *et al.* 2004). Indeed, biochemical *in vitro* studies revealed K_m values of purified PHDs for oxygen close to the oxygen partial pressure (pO_2) in air, suggesting that the kinetics of specific HIFα hydroxylation under hypoxic conditions are rather slow (Hirsilä *et al.* 2003; Ehrismann *et al.* 2006). However, tissues *in situ* have to deal with a great variability of generally very low pO_2 values, even when the inspiratory pO_2 is considered to be “normoxic”. We recently showed that HIF-dependent regulation of PHD levels adapts the PHD-HIF oxygen sensing system to a given tissue pO_2, rather than simply accelerating HIFα destruction following re-oxygenation (Stiehl *et al.* 2006). Such a self-regulatory loop might define a tissue-specific threshold for HIFα-activation as a function of local pO_2.

In addition to transcriptional regulation, PHD levels are also regulated by protein-protein interactions: the ubiquitin ligase Siah2 regulates PHD1 and PHD3, but not PHD2, protein stability (Nakayama *et al.* 2004); PHD3, but not PHD1 or PHD2, appears to be a substrate for the TRiC chaperonin (Masson *et al.* 2004); OS-9 apparently is simultaneously interacting with both HIFα and PHD2 or PHD3, but not PHD1, thereby enhancing HIFα hydroxylation and degradation (Baek *et al.* 2005); MORG might provide the molecular scaffold for HIFα interaction specifically with PHD3 (Hopfer *et al.* 2006); and ING4, a likely component of a chromatin-remodeling complex, might be recruited via PHD2 to enhance HIF transcriptional activity (Ozer *et al.* 2005). On the other hand, PHD2 has been shown to inhibit the transactivation function of HIF-1α in VHL-deficient cells (To and Huang 2005)

These findings suggest two additional layers in oxygen signalling: first, abundance and function of PHDs can indeed be regulated; and second, the three different PHDs are regulated in non-identical ways, further supporting

their non-redundant roles in oxygen sensing. In fact, while all three PHDs can hydroxylate HIFα with similar efficiency, PHD2 has been suggested to play the main role for normoxic HIFα turnover (Berra *et al.* 2003). Consistent with these *in vitro* findings, PHD2 but neither PHD1 nor PHD3 knock-out mice die during embryonic development (Takeda *et al.* 2006). Interestingly, a family with erythrocytosis due to a mutation in the gene encoding PHD2 has recently been reported (Percy *et al.* 2006). The three PHDs are expressed in most organs but there are strikingly high levels of PHD3 mRNA expressed in the heart and of PHD1 mRNA expressed in the testis (Stiehl *et al.* 2006; Willam *et al.* 2006)

PHD function can also be regulated by a number of endogenous small molecules as well as by a number of clinically relevant drugs: ascorbate (Knowles *et al.* 2003), transition metals (Hirsila *et al.* 2005; Martin *et al.* 2005), Krebs cycle intermediates (Dalgard *et al.* 2004; Lu *et al.* 2005; Selak *et al.* 2005) and reactive oxygen species (ROS) including NO (Metzen *et al.* 2003; Gerald *et al.* 2004; Berchner-Pfannschmidt *et al.* 2006) has been shown to influence or completely block the activity of the PHDs. Thus, there appear to exist molecular cross-talks between oxygen homeostasis and transition metal homeostasis as well as cellular metabolism. However, it is not completely understood how various transition metals and ascorbate interfere with PHD and FIH function. The transition metals do not simply replace or oxidize the ferrous iron in the active center of the hydroxylases (Hirsila *et al.* 2005; Martin *et al.* 2005). More likely, transition metals might decrease the availability of ascorbate by catalysing oxygen-dependent ascorbate oxidation (Salnikow *et al.* 2004; Karaczyn *et al.* 2006). As known for collagen hydroxylation, ascorbate might be required for reducing ferric iron in the active center of PHDs and FIH which occurs when oxidative decarboxylation of 2-oxoglutarate is uncoupled from target hydroxylation (Myllyla *et al.* 1984; McNeill *et al.* 2005)

In addition to kinase signalling pathways, numerous reports appeared about an involvement of ROS in HIFα protein stabilization by either growth stimuli or hypoxia. Non-conclusive data were obtained so far about the source(s) of the ROS and whether hypoxia leads to an increase or decrease

in ROS levels (Wenger 2000). However, ROS do have the potential to interfere with the complex process of protein hydroxylation. Indeed, the increase in ROS in *junD*$^{-/-}$ cells leads to a decrease in PHD activity and hence to HIF-1α accumulation (Gerald *et al.* 2004). Apart from direct interference of ROS with the active center of oxygen sensing protein hydroxylases, also the re-direction of oxygen from mitochondria towards the protein hydroxylases might contribute to these effects (Wenger 2006)

Due to the large therapeutical potential of PHD inhibitors in the treatment of anemic and ischemic diseases, several attempts to develop PHD antagonists are in progress. Currently, N-oxyalylglycine (N-OG) and its cell-permeable derivative dimethyloxalylglycine (DMOG) are the commercially available PHD inhibitors of choice for experimental purposes. Apart from these co-substrate competitive inhibitors, iron chelators are very well known to induce HIF by PHD inhibition. These include desferoxamine (DFX) and ciclopirox olamine (CPX), two clinically relevant hydroxamic acid iron chelators (Linden *et al.* 2003). In order to functionally investigate the potency and mechanisms of available and future PHD inhibitors, there is a clear need for reliable methods to generate PHD proteins and to determine their activity.

2.1.2.1 Production of functionally active PHDs

Since bacterially expressed PHDs retain only little hydroxylase activity, PHDs were expressed and purified from baculovirally infected insect cells. To facilitate purification, the PHDs were tagged with either glutathione-S-transferase (GST) or His_6. According to our experience, best results were obtained with GST-tagged PHD proteins. Thus, GST-tagged expression vectors were prepared by LR clonase-mediated homologous recombination of the corresponding pENTR vector, containing PHD1, PHD2 or PHD3 cDNA inserts, with the pDEST20 vector (Invitrogen). Recombined plasmids were transfected into the *E. coli* strain Top10 and the resulting bacmid plasmids were used to generate baculovirus stocks according to the manufacturer's instructions (Invitrogen). *Spodoptera frugiperda* (Sf) 9 cells were infected with baculovirus and cultured in Grace's insect medium (Invitrogen) at 25°C in a humidified incubator. Infected cells were grown for 80 to 110 hours, collected

by centrifugation at 700 × g for 10 minutes at 4°C and washed with ice-cold PBS. Lysis was performed on ice for 10 minutes with 0.1% NP-40 in a buffer containing 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 100 mM glycine and 100 μM dithiothreitol (DTT). Crude lysate was cleared by centrifugation at 20,000 × g for 20 minutes at 4°C and the supernatant was incubated with glutathione-sepharose beads (previously washed with PBS) for at least 2 hours at 4°C with gentle agitation. After washing three times with PBS the protein was eluted with 15 mM reduced glutathione (GSH) in 50 mM Tris-HCl pH 8.0, 5% glycerol and 2 μM $FeSO_4$. Purity was checked by SDS-PAGE followed by coomassie staining or immunoblotting (Fig. 1B). Activity was routinely determined by the VHL binding assay described below (Fig. 1C).

2.1.2.2 Determination of prolyl-4-hydroxylation by VHL binding to peptides derived from the HIF-1α ODD domain

Enzymatic activities of recombinant PHD proteins were determined by an *in vitro* hydroxylation assay based on a 96-well format as described previously (Oehme *et al.* 2004; Martin *et al.* 2005). Briefly, biotinylated synthetic peptides derived from human HIF-1α amino acids 556 to 574 (biotin-DLDLEALAPYIPADDDFQL; either wild-type, P564A mutant or Hyp564 modified) were bound to NeutrAvidin-coated 96-well plates (Pierce). All methionine residues were mutated to alanine residues in these peptides (M561A, M568A). Hydroxylase reactions using purified recombinant GST-tagged PHDs or cellular extracts were carried out for 1 hour at room temperature. A polycistronic bacterial expression vector for His_6-tagged and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex (kindly provided by S. Tan, Pennsylvania, PA) was used to express VBC in *E. coli* strain BL21AI followed by purification using nickel affinity chromatography (Amersham-GE Healthcare). The VBC complex was allowed to bind to the hydroxylated peptides for 15 minutes, anti-thioredoxin antibodies were added for 30 minutes, and horseradish peroxidase-coupled anti-rabbit antibodies (Sigma) were added for 30 minutes. Bound VBC complex was detected using the TMB (3,3',5,5'-tetramethylbenzidine) substrate kit (Pierce). The peroxidase reaction was stopped by adding one volume 2 M H_2SO_4 and

absorbance was determined at 450 nm in a microplate photometer. This assay is routinely used to determine the activity of the three GST-tagged PHD enzymes purified from Sf9 insect cells (Fig. 1C). Inter-assay comparability was guaranteed by calibration of each experiment to an internal standard curve using increasing fraction of synthetic hydroxyproline-containing peptides (Fig. 2A). When performed with pre-equilibrated solutions in a hypoxic glove box (Invivo2 400, Ruskinn Technology), this assay nicely demonstrates that PHD2 and PHD3 hydroxylase activities are functional over a wide range of physiologically relevant oxygen concentrations (Fig. 2B). Furthermore, this assay is strictly dependent on the presence of P564 within the substrate peptide, and the iron chelator DFX as well as the substrate analog N-OG inhibit PHD-dependent prolyl-4-hydroxylation (Fig. 2C).

Apart from purified recombinant PHDs, also crude cellular extracts are suitable sources of PHD activity for the VBC binding assay. Therefore, Hep3B cells were lysed by dounce homogenization in 100 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 8.75% glycerol, 0.01% Tween20 and EDTA-free protease inhibitor cocktail (Roche). Cell lysates were cleared by centrifugation at 20,000 $\times$ g for 30 minutes at 4°C. As shown in Fig. 2D, these extracts stimulated VBC binding to wild type but not P564A mutant HIF-1αODD-derived peptides. The specificity of the PHD activity was further demonstrated by inhibition with N-OG and by culturing the Hep3B cells under hypoxic conditions (0.4% O_2 for 16 hours). Consistent with the known hypoxic induction of PHD2 and PHD3, extracts derived from hypoxic Hep3B cells showed an increased PHD activity (Fig. 2D). Similar results were obtained with HeLa cells (data not shown).

2.1.2.3 Determination of prolyl-4-hydroxylation by oxidative decarboxylation of 2-oxoglutarate

While the VHL binding assay is well suited to investigate PHD function, it is strictly dependent on HIFα-derived peptides as hydroxylation substrates and hence does not allow to investigate the hydroxylation of potential novel non-HIFα targets. To overcome this problem, a 2-oxoglutarate to succinate conversion assay, originally developed to study collagen hydroxylation (Kaule and Gunzler 1990), was adapted to HIFα hydroxylation. There are basically

two possibilities to label the 2-oxoglutarate co-substrate: when [5-^{14}C]2-oxoglutarate is used, the resulting [^{14}C]succinate can be measured (D'Angelo *et al.* 2003; D'Angelo *et al.* 2003); when [1-^{14}C]2-oxoglutarate is used, the resulting [^{14}C]CO_2 must be captured and quantified (Hirsila *et al.* 2003). Because it is technically less demanding, the former technique is used in our laboratory. This assay was first applied for PHD measurements by Frelin and co-workers using "light mitochondrial rat kidney fractions" (D'Angelo *et al.* 2003; D'Angelo *et al.* 2003). We measured oxidative decarboxylation of 2-oxoglutarate by purified PHDs, because kidney extracts prepared according to Frelin and co-workers in fact solely showed high background activities, but did not contain measurable quantities of specific PHD activity (see below). Peptide or protein substrates (about 5 μM) were incubated with 0.1 μg GST-tagged PHD enzymes in 100 μl 50 mM Tris-HCl pH 7.4, 10 μM 2-oxoglutarate, 2 mM ascorbate, 100 μM DTT, 1 mg/ml BSA, 0.6 mg/ml catalase, 5 μM freshly prepared $FeSO_4$ and 50,000 - 100,000 dpm [5-^{14}C]2-oxoglutarate with a specific activity of 50 mCi/mmol (Hartmann-Analytic). Hydroxylation reactions were carried out for 24 hours at 37°C. To separate 2-oxoglutarate from succinate, 25 μl 20 mM succinate and 20 mM 2-oxoglutarate were added and mixed by vortexing. Subsequently, 25 μl 0.16 M 2,4-dinitrophenyl hydrazine in 30% $HClO_4$ were added. After incubation at room temperature for 30 minutes, 50 μl of 1 M 2-oxoglutarate was added to remove residual 2,4-dinitrophenyl hydrazine, and the reaction was allowed to proceed for another 30 minutes at room temperature. Following centrifugation at 20,000 × g for 15 minutes at 4°C, 150 μl supernatant was carefully removed, mixed with 3 ml scintillation cocktail (PerkinElmer), and the amount of [^{14}C]succinate was determined by liquid scintillation counting in a β-counter (TRI-CARB 2900TR, Packard).

As depicted in Fig. 3A, using recombinant GST-PHD2 this assay shows a linear relationship between the availability of a HIF-1α ODD domain-derived synthetic peptide substrate and [^{14}C]succinate production in the range from 8 to 50 μM peptide. Below 5 μM, however, only background values were obtained. This assay is strictly dependent on the presence of 1 mM ascorbate

as well as a peptide substrate containing the wild-type P564 (Fig. 3B). The lack of a substrate peptide or the mutation of the critical proline (P564A) results in background 2-oxoglutarate conversion.

2.1.2.4 Crude tissue extracts are not a suitable source of PHD activity for the 2-oxoglutarate conversion assay.

Earlier reports suggested the use of "light mitochondrial rat kidney fractions" as a source for PHD activity in this type of assay (D'Angelo *et al.* 2003; D'Angelo *et al.* 2003). We therefore prepared rat kidney extracts according to this protocol and tested them for PHD activity. As shown in Fig. 4A, 2-oxoglutarate conversion was indeed stimulated by these extracts. However, a very high background activity was also observed in the absence of the HIF-1α ODD domain-derived peptide and the presence of the wild-type peptide stimulated 2-oxoglutarate conversion only moderately. To ensure PHD-dependent 2-oxoglutarate conversion, ample amounts of the known PHD inhibitors DFX and N-OG (see Fig. 2C) or $CuCl_2$ (Martin *et al.* 2005) were added to the reaction mix. Surprisingly, none of these PHD inhibitors blocked 2-oxoglutarate conversion, whether the substrate peptide was present or not (Fig. 4A, left panel). Moreover, the presence of a P564A mutant peptide stimulated 2-oxoglutarate conversion as well as the wild-type peptide (Fig. 4A, right panel). The P564A mutant peptide cannot be hydroxylated and shows no VBC complex binding after incubation with purified PHDs (Figs. 2C and 3B). Therefore, these experiments clearly demonstrate that "light mitochondrial rat kidney fractions" do not represent a source of specific PHD activity but rather contain high levels of enzymes unspecifically metabolising 2-oxoglutarate. A similar lack of specific (i.e. PHD-dependent) 2-oxoglutarate conversion was observed with crude extracts derived from HeLa, Hep3B/Hek293 and Sf9 cells (data not shown).

2.1.2.5 Thin layer chromatography to assess the purity of [5-^{14}C]2-oxoglutarate

A major problem in the optimisation of the 2-oxoglutarate conversion method is the high proportion of background activity when non-purified PHD

preparations are used as source of the enzymatic activity. Indeed, cellular extracts usually contain too high background activities to be suitable as PHD source, even when the PHDs are exogenously overexpressed. Another background-causing problem was the quality of the radioactively labelled [5-^{14}C]2-oxoglutarate. To analyse the [5-^{14}C]2-oxoglutarate preparations for impurities, it was diluted in 1.5 mM non-labeled 2-oxoglutarate and spotted onto thin layer chromatography (TLC) plates (Silica gel 60 F254, Merck). Following drying, the TLC plate was placed in a chromatography chamber containing a 120:70:15 mixture of diethyl ether/hexane/formic acid. When the eluent reached the top of the TLC plate, it was dried and the radioactivity quantitated by phosphorimaging (BioRad). Non-labeled 2-oxoglutarate and succinate were used as standards. They were visualized as bright yellow spots by immersing the TLC plates in a 0.04% bromcresol purple solution in a 1:1 mixture of ethanol and water (adjusted to pH 10.0 with NaOH) and dried with a hair-dryer. R_f values for succinate and 2-oxoglutarate were 0.45 and 0.12, respectively. As shown in Fig. 4B, there are considerable differences in the quality of the available [5-^{14}C]2-oxoglutarate preparations. While some batches were of acceptable purity (Fig. 4B, left panel), others contained up to 30% of an impurity that co-migrated with the succinate standard (Fig. 4B, right panel). Regarding the relatively low specific PHD activities and the rather high unspecific background 2-oxoglutarate conversion, such high impurities are not acceptable in this type of assay.

2.1.2.6 Application of the 2-oxoglutarate conversion assay to protein targets

In order to be useful for putative novel PHD substrate proteins without prior knowledge of the actual target prolyl residue, the 2-oxoglutarate conversion assay needs to work also with proteins rather than only with synthetic peptides. To demonstrate the feasibility of this approach, wild-type GST-HIF2αODD (aa 404-569, MW = 46 kDa) and P405A/P531A double-mutant GST-HIF2αODD protein fragments were expressed in *E.coli* BL21AI by induction with 0.2% arabinose for 4 hours at 37°C. After harvesting by centrifugation, the bacteria were lysed with a high pressure cell disrupter

(Basic-Z, Constant Systems) in the prescence of an EDTA-free protease inhibitor cocktail (Roche). Purification of the GST-tagged protein was carried out using affinity chromatography on glutathione sepharose beads (Amersham-GE Healthcare) as described above (see Fig. 1B). Purity of the recombinant protein fragments was checked by SDS-PAGE followed by coomassie staining or immunoblotting (Fig. 5A). When compared with a HIF-1αODD-derived wild-type peptide, equimolar concentrations of the HIF2αODD protein fragment also stimulated PHD1-dependent 2-oxoglutarate conversion, albeit to a somewhat lower extent (Fig. 5B).

2.1.3 Conclusions

The PHD-dependent hydroxylation assay presented here works independently of already known peptide sequences or VHL binding. It should thus be possible to use this assay for the investigation of putative novel, non-HIFα PHD substrates as well. In addition, even if not shown here, the same assay should principally also be applicable to novel FIH substrates and might become useful for screening for novel hydroxylation targets. Whereas no non-HIFα PHD targets have been reported thus far, FIH has recently been shown to hydroxylate a number of proteins containing ankyrin repeats, including NF-κB and IκBα (Cockman *et al.* 2006). We expect a similar widening of the spectrum of PHD targets. In addition, PHD-dependent hydroxylation assays will be required to study novel drugs that modulate PHD activity and hence will become important for the treatment of anemic and ischemic disease.

2.1.4 Acknowledgments

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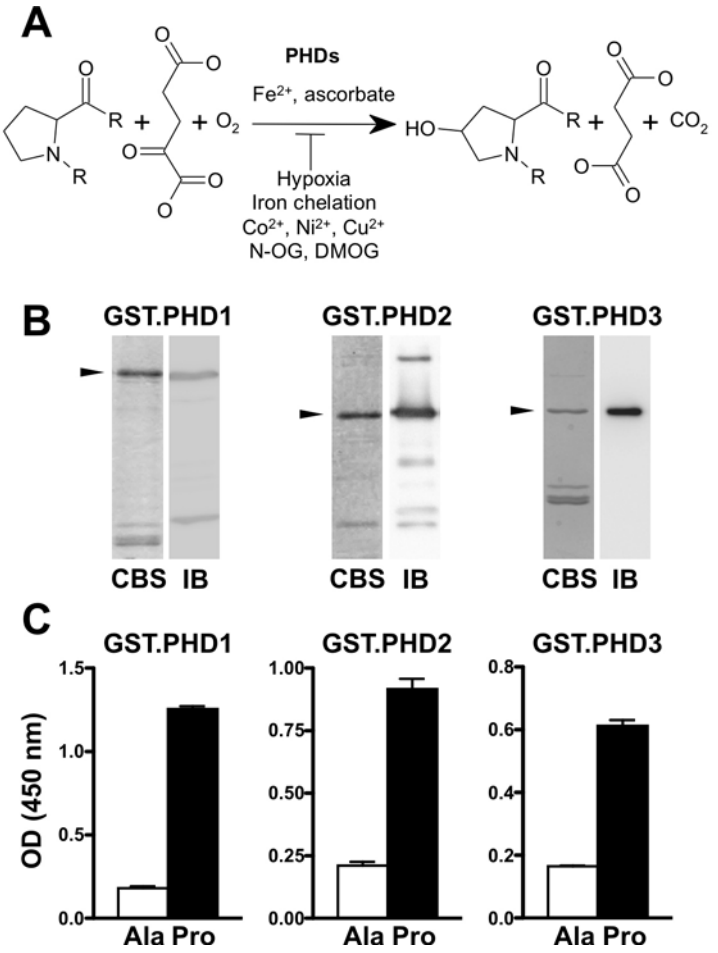

Figure 1. (A) Reaction mechanism of PHD-mediated oxygen-dependent prolyl-4-hydroxylation by oxidative decarboxylation of the co-substrate 2-oxoglutarate. Inhibitors of this reaction are indicated. N-OG, N-oxalylglycine; DMOG, dimethyloxalylglycine. (B) Purification of GST-tagged PHD1, PHD2 and PHD3 expressed in baculovirus-infected Sf9 insect cells by glutathione affinity chromatography. The purified GST-PHD proteins were analyzed by SDS-PAGE and coomassie blue staining ("CBS") or immunoblotting ("IB") using anti-GST antibodies. (C) Hydroxylation activities of GST-PHD preparations determined by a VBC-binding assay as described in the text. Wild-type ("Pro") and P564A mutant ("Ala") HIF-1α ODD domain-derived peptides were used as hydroxylation substrates.

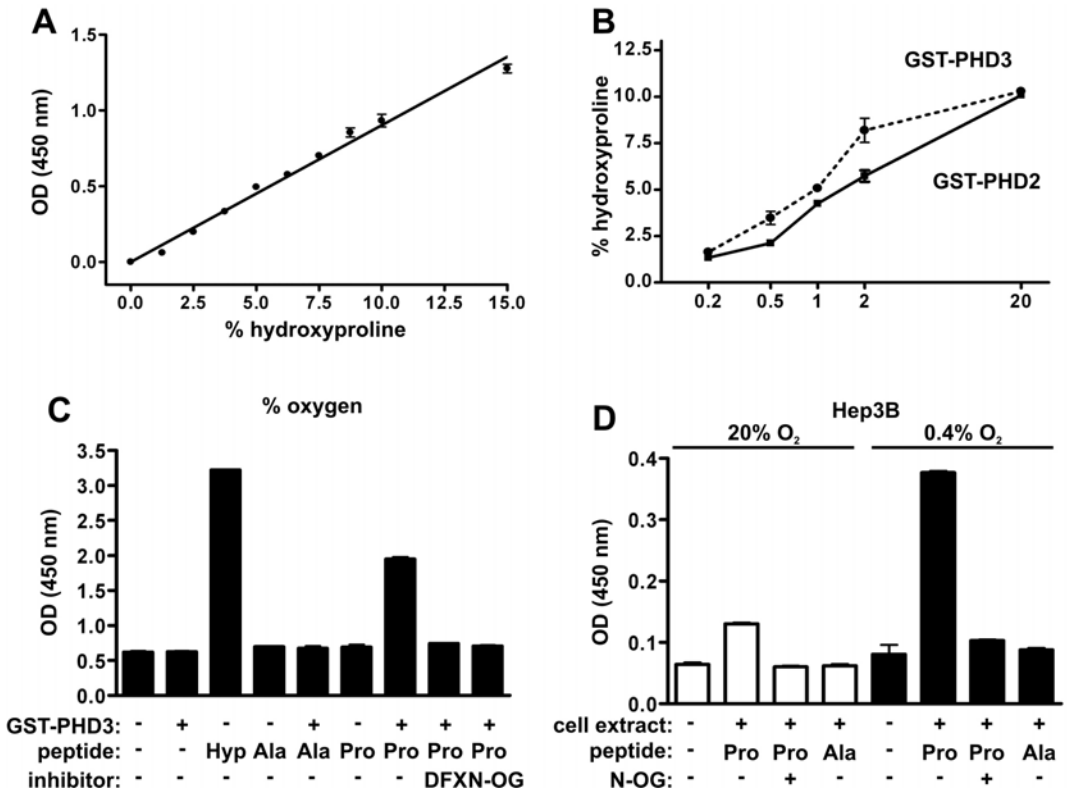

Figure 2. (A) VBC (pVHL/elongin B/elongin C) complex binding to prolyl-4-hydroxylated HIF-1α ODD domain-derived peptides. Biotinylated peptides containing increasing proportions of synthetically generated hydroxyproline peptides were allowed to bind to neutravidin-coated 96 well ELISA plates. Immunodetection of bound VBC complex was performed as described in the text. (B) Oxygen-dependent activity of recombinant GST-PHD2 and GST-PHD3. Purified PHD proteins were used to hydroxylate HIF-1α ODD domain-derived peptides under the indicated oxygen concentrations (% vol/vol in the gas phase). The extent of prolyl-4-hydroxylation was quantitated as described above. (C) PHD3 as well as the wild-type peptide substrate ("Pro") are required for VBC binding. The lack of peptide ("-"), a P564A mutation ("Ala"), iron chelation by desferrioxamine ("DFX") or N-oxalylglycine ("N-OG") all completely inhibited VBC binding. (A-C) Shown are mean values ± SEM of triplicates. (D) Hypoxia stimulates PHD activity in Hep3B cells. Crude cellular lysates were used to hydroxylate HIF-1α ODD domain-derived peptides as described above. Shown are mean values ± SEM of triplicates normalized to protein content.

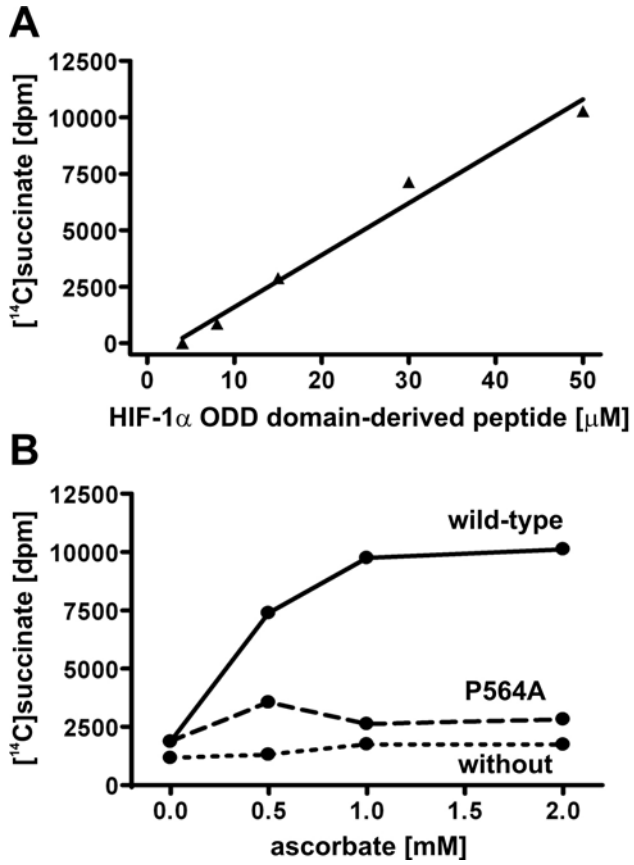

Figure 3. (A) Determination of prolyl-4-hydroxylation by oxidative decarboxylation of [5-^{14}C]2-oxoglutarate. Recombinant GST-PHD2 was incubated with increasing concentrations of a HIF-1α ODD domain-derived peptide as indicated. PHD-dependent generation of [^{14}C]succinate was determined as described in the text. Background activity was determined in control reactions without substrate peptide and the obtained value subtracted from the peptide-containing reactions. (B) Recombinant GST-PHD2 was incubated with increasing concentrations of ascorbate as indicated and either a wild-type, P564A mutant or no peptide substrate.

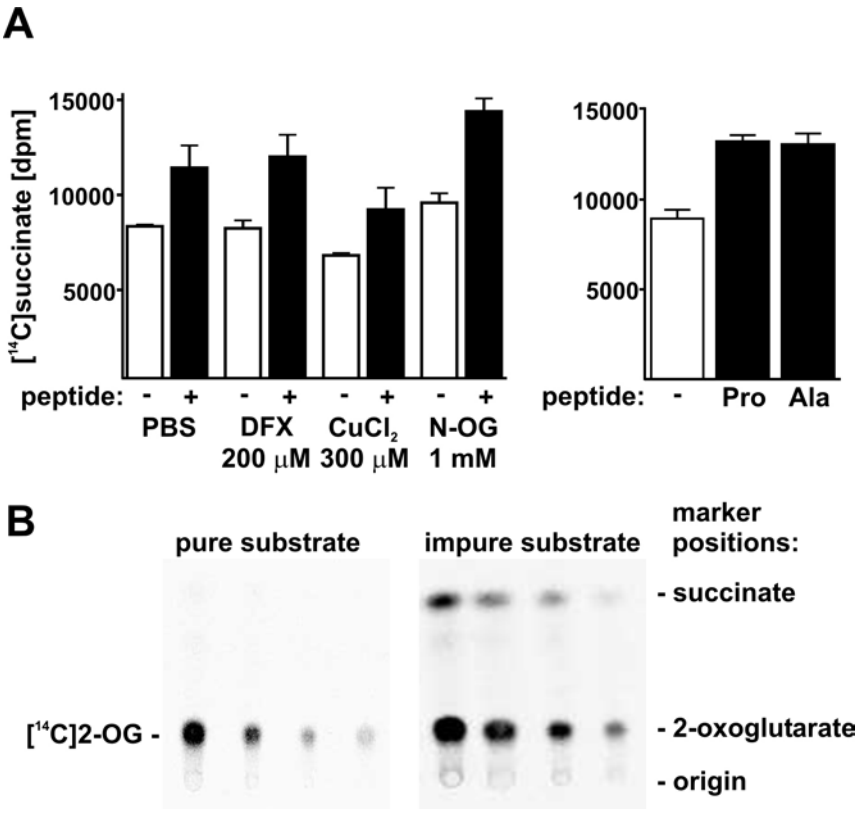

Figure 4. (A) Lack of specific 2-oxoglutarate-to-succinate conversion by "light mitochondrial rat kidney fractions". Left panel: peptide-stimulated activity in these extracts could not be inhibited by DFX, CuCl2 or N-OG. Right panel: a P564A mutant ("Ala") HIF-1α ODD domain-derived peptide stimulated 2-oxoglutarate conversion as well as the wild-type ("Pro") peptide. (B) Quality control of [5-^{14}C]-2-oxoglutarate by thin layer chromatography. The amount of [5-^{14}C]-2-oxoglutarate spotted corresponded to 30 kdpm, 15 kdpm, 7.5 kdpm and 3.8 kdpm, respectively (from left to right). Two [5-^{14}C]-2-oxoglutarate batches of different quality are shown. Pure 2-oxoglutarate and succinate served as migration markers (positions indicated at the right margin).

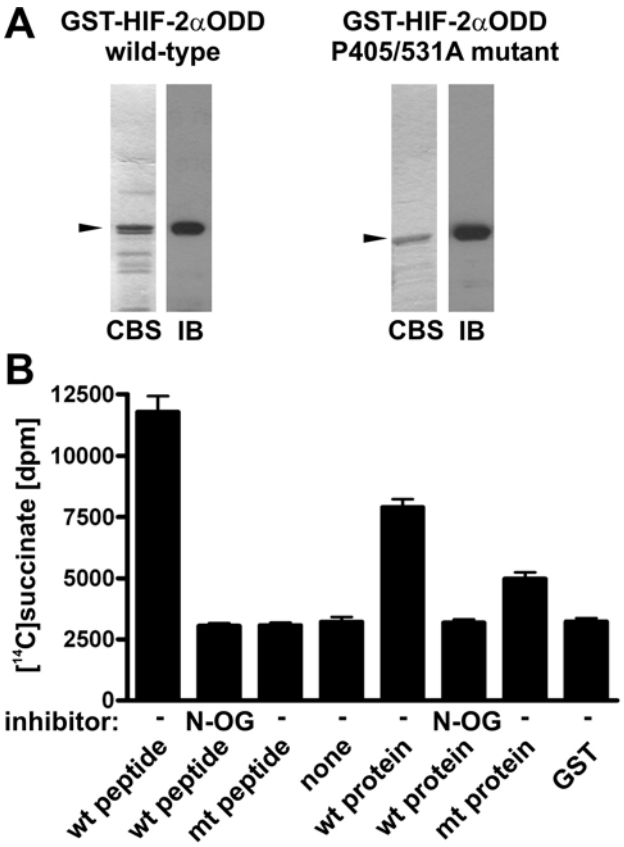

Figure 5. (A) Purification of wild-type and P405/531A double mutant GST-HIF-2α ODD (human HIF-2α amino acids 404-569) protein fragments expressed in bacteria and purified by glutathione affinity chromatography. The purified proteins were analyzed by SDS-PAGE and coomassie blue staining ("CBS") or immunoblotting ("IB") using anti-GST antibodies (B) Determination of prolyl-4-hydroxylation by oxidative decarboxylation of [5-^{14}C]2-oxoglutarate. Recombinant GST-PHD1-dependent generation of [^{14}C]succinate was promoted by HIF-1αODD-derived wild-type ("wt") but not by P564A mutant ("mt") peptides (human HIF-1α amino acids 556-574). Generation of [^{14}C]succinate was also promoted by wild-type GST-HIF-2αODD protein but not by GST alone and to a much lower extent by the GST-HIF-2αODD P405/531A double mutant. Equimolar concentrations (5 μM) of the substrate peptides and protein fragments were used. Where indicated, the reactions could be blocked by 5 mM N-oxalylglycine ("N-OG"). Shown are mean values ± SEM of three independent experiments performed in duplicates.

2.2 Increased prolyl-4-hydroxylase domain (PHD) proteins compensate for decreased oxygen levels: evidence for an autoregulatory oxygen sensing system

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2.2.1 Abstract

Prolyl-4-hydroxylase domain (PHD) proteins are oxygen-dependent enzymes that hydroxylate hypoxia-inducible transcription factor (HIF) α-subunits, leading to their subsequent ubiquitinylation and degradation. Paradoxically, the expression of two family members (PHD2 and PHD3) is induced in hypoxic cell culture despite the reduced availability of oxygen co-substrate, and it has been suggested that they become functionally relevant following re-oxygenation to rapidly terminate the HIF response. Here we show that PHDs are also induced in hypoxic mice in vivo, albeit in a tissue-specific manner. As demonstrated under chronically hypoxic conditions in vitro, PHD2 and PHD3 show a transient maximum but remain upregulated over more than 10 days, suggesting a feedback downregulation of HIF-1α which then levels off at a novel setpoint. Indeed, hypoxic induction of PHD2 and PHD3 is paralleled by the attenuation of endogenous HIF-1α. Using an engineered oxygen-sensitive reporter gene in a cellular background lacking endogenous HIF-1α and hence inducible PHD expression, we could show that increased exogenous PHD levels can compensate for a wide range of hypoxic conditions. Similar data were obtained in a reconstituted cell-free system in vitro. In summary, these results suggest that due to their high O_2 Km values PHDs have optimal oxygen-sensing properties under all physiologically relevant oxygen concentrations: increased PHDs play a functional role even under oxygen-

deprived conditions, allowing the HIF system to adapt to a novel oxygen threshold and to respond to another hypoxic insult. Furthermore, such an autoregulatory oxygen-sensing system would explain how a single mechanism works in a wide variety of differently oxygenated tissues.

2.2.2 Introduction

Biological systems tightly monitor acute changes in environmental conditions, initiate regulatory responses, and use negative feedback loops to limit the extent of these responses. To adapt to chronic changes, many environmental sensors are capable of adjusting their threshold values, allowing to respond again to acute deviations of a now different setpoint.

Cells sense changes in environmental oxygen availability by a group of enzymes which directly control the cellular response to lowered oxygen by destabilizing hypoxia-inducible factor (HIF) α subunits, the master transcriptional regulators of the hypoxic response. These oxygen-sensing enzymes have alternatively been termed prolyl-4-hydroxylase domain (PHD), HIF prolyl hydroxylase (HPH) or egg laying defective nine homolog (EGLN). Three family members are known up to date: PHD1/HPH3/EGLN2, PHD2/HPH2/EGLN1 and PHD3/HPH1/EGLN3 (Bruick 2000; Epstein *et al.* 2001; Ivan *et al.* 2002). PHDs hydroxylate HIF-1α and HIF-2α at two distinct proline residues within the HIFα oxygen-dependent degradation (ODD) domain. Under normoxic conditions, prolyl-4-hydroxylation allows binding of the von Hippel-Lindau tumor suppressor protein (pVHL), leading to polyubiquitinylation and proteasomal destruction (Wenger 2002). Under hypoxic conditions, prolyl-4-hydroxylation is reduced, HIF-1α and HIF-2α become stabilized, heterodimerize with the constitutively expressed HIF-1β subunit aryl hydrocarbon receptor nuclear translocator (ARNT), and regulate the expression of a large number of effector genes involved in adaptation to low oxygen (Wenger *et al.* 2005). In addition, factor inhibiting HIF (FIH) hydroxylates a C-terminal asparagine residue, thereby regulating the transcriptional activity of HIFs (Mahon *et al.* 2001; Hewitson *et al.* 2002; Lando *et al.* 2002).

Upon re-oxygenation, the PHD oxygen sensing system must be rapidly reversed. Interestingly, PHD2 and PHD3, but not PHD1, have been reported to be hypoxically induced at both the mRNA and protein levels (Epstein *et al.* 2001). Accordingly, elevated PHD2 and PHD3 levels have been demonstrated in a broad panel of established cancer cell lines (Appelhoff *et al.* 2004). Functional hypoxia response elements (HREs) are located in the promoter region of the human *PHD2* gene as well as in the first intron of the human *PHD3* gene, suggesting that *PHD2* and *PHD3* are HIF target genes themselves (Metzen *et al.* 2005; Pescador *et al.* 2005). Because the essential co-factor oxygen is basically lacking under hypoxic conditions, the HIF-dependent hypoxic increase in PHD abundance has been suggested to play a role in accelerating the termination of the HIF response following re-oxygenation (Epstein *et al.* 2001; Appelhoff *et al.* 2004; Aprelikova *et al.* 2004; Marxsen *et al.* 2004). Indeed, biochemical *in vitro* studies revealed K_m values of purified PHDs for oxygen close to the oxygen partial pressure (pO_2) in air, suggesting that the kinetics of specific HIFα hydroxylation under hypoxic conditions are rather slow(Hirsilä *et al.* 2003). However, tissues *in situ* have to deal with a great variability of generally very low pO_2 values, even when the inspiratory pO_2 is considered to be “normoxic”. We therefore raised the question, whether HIF-dependent regulation of PHD levels might lead to the adaptation of the PHD-HIF oxygen sensing system to a given tissue pO_2, rather than simply accelerating HIFα destruction following re-oxygenation. Such a self-regulatory loop might define a tissue-specific threshold for HIFα-activation as a function of local pO_2.

2.2.3 Materials and Methodes

2.2.3.1 Cell culture

All cells were maintained in DMEM high glucose, containing 4.5 mg/ml glucose (Sigma, Buchs, Switzerland) to maintain cellular energy metabolism during prolonged hypoxic culturing. Culturing media were supplemented with 10% heat-inactivated fetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Basel, Switzerland). Mouse embryonic fibroblast

(MEF)-*Hif1a*$^{+/+}$ and MEF-*Hif1a*$^{-/-}$, and mouse hepatoma Hepa1 and Hepa1c4 were cultured as described before (Chilov *et al.* 1999; Unruh *et al.* 2003). For long-term hypoxia, MEFs were grown under 2% O_2 for up to 256 hours in a gas-controlled glove box to allow for handling of the cells under constant pO_2 (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Cells were grown on 145 mm culture dishes and splitted every 48 hours. Reagents used for splitting and permanent culturing were pre-equilibrated to the pO_2 in the glove box before use. For oxygen titration, a single batch of transfected MEF-*Hif1a*$^{-/-}$ cells was distributed into different hypoxic incubators (Binder, Tuttlingen, Germany) and simultaneously cultured for the indicated time periods.

2.2.3.2 Protein extractions and immunoblot analyses

Cells were washed twice and scraped into ice-cold PBS. Soluble cellular protein was extracted with a high-salt extraction buffer containing 0.1% NP-40 essentially as described before (Martin *et al.* 2005). Protein concentrations were determined by the Bradford method and 50 to 80 μg protein was subjected to immunoblot analysis. Antibodies were obtained from the following sources: anti-human HIF-1α mouse monoclonal antibody (mAb), Transduction Laboratories (BD Biosciences, Basel, Switzerland); anti-human PHDs rabbit polyclonal antibodies, Novus Biologicals (Littleton, CO); anti-V5-tag mAb, Invitrogen; anti-β-actin mAb, Sigma; Horseradish peroxidase-coupled secondary antibodies, Pierce (Perbio, Lausanne, Switzerland). Anti-human PHD3 mAb was kindly provided by P.J. Ratcliffe (Oxford, UK). Bound antibodies were detected with ECL substrate (Pierce) and chemiluminescence was quantified with a CCD-camera-based light imaging system (FluorChem8900, AlphaInnotech, Witec, Littau, Switzerland) using QuantityOne software (Biorad, Reinach, Switzerland).

2.2.3.3 mRNA quantification

Exposure of mice to inspiratory 0.1% carbon monoxide or 7.5% oxygen and serum erythropoietin quantification by RIA have been published previsously (Wenger *et al.* 1996; Wenger *et al.* 1998; Wenger and Gassmann 1999). Total RNA from tissue and cells was purified as described previously (Martin *et al.*

2005). RNA concentrations were determined spectrophotometrically and RNA integrity was monitored by denaturing formaldehyde/agarose gel electrophoresis. Total RNA (5 μg) was reverse transcribed (RT) using oligo-dT and Stratascript reverse transcriptase (Stratagene, LaJolla, CA). mRNA levels for mouse carbonic anhydrase (CA) IX, glucose transporter (GLUT) 1, erythropoietin, PHD1, PHD2 and PHD3 were quantified with 2 μl of diluted cDNA reaction (corresponding to 1% of cDNA reaction) by real-time RT-PCR. A SybrGreen qPCR reagent kit (Sigma) was used in combination with a MX3000P light cycler (Stratagene). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein S12 mRNA was determined and data expressed as ratios relative to S12 levels. Melting point analyses of amplified PCR products were performed after each run to verify specific amplification. For primer sequences see Supplemental Data.

2.2.3.4 Plasmid constructions

Plasmids encoding full-length mouse HIF-1α, both wild-type and P402A and/or P563A mutants (note that all amino acid numbering correspond to mouse HIF-1α sequences), were a kind gift of L. Poellinger (Stockholm, Sweden). A PCR-product spanning the HIF-1α ODD region (amino acid 359 to 685) was cloned into the *Eco*RI site of pM3-VP16 (Clontech, BD Biosciences) to obtain one-hybrid constructs harboring a N-terminal GAL4 DNA-binding domain and a C-terminal VP16 transactivation domain. Resulting plasmids were termed G4.mHIF(359-685).VP16_wt, G4.mHIF(359-685).VP16_P402A, G4.mHIF(359-685).VP16_P563A and G4.mHIF(359-685).VP16_PP/AA. For overexpression in mammalian cells, full-length human PHD2 and PHD3 (kindly provided by W. Kaelin Jr., Boston, MA, and I. Flamme, Wuppertal, Germany, respectively) were subcloned into pENTR4 and recombined into pcDNA3.1/nV5-DEST using Gateway technology (Invitrogen). Similarly, vectors for expression of GST-tagged PHD isoforms in Sf9 insect cells were constructed by recombining coding sequences for PHD2 and PHD3 into pDEST20 (Invitrogen). All primary cDNA inserts were sequenced (Microsynth, Balgach, Switzerland).

2.2.3.5 Transient transfections

Cells were co-transfected with the indicated amounts of DNA using polyethylenimine (PEI, Polysciences, Warrington, PA). Therefore, cells were grown on 100 mm dishes to subconfluency and 200 µl 150 mM NaCl containing a DNA-PEI mixture (1:5, w/w) was added. For co-transfections, total DNA was kept constant by adding empty vector. Following over-night incubation, the cells were trypsinized and divided onto 12-well plates for luciferase assays and 100 mm dishes for immunoblotting.

2.2.3.6 Luciferase assays

MEF-*Hif1a*$^{-/-}$ cells were transiently co-transfected with 2 µg of indicated G4.mHIF(359-685).VP16 fusion constructs and up to 8 µg of the respective V5.PHD isoform, along with 1 µg of the GAL4-responsive reporter plasmid pGRE5xE1b, containing the firefly luciferase gene under control of E1b promoter and five GAL4 response elements (kind gift of D. Peet, Adelaide, Australia). Identical amounts of total DNA were transfected in each experimental setting. Following transfection, cultures were grown for additional 24 hours at the indicated oxygen concentration and lysed in 100 µl passive lysis buffer (Promega, Madison, WI). Luciferase reporter gene activity was determined in a microplate luminometer (Berthold, Regensdorf, Switzerland) using luciferase firefly substrate (Promega). Protein concentration in the lysates was determined by a Bradford assay and relative luciferase activity was calculated from the ratio between relative light units and µg protein.

2.2.3.7 RNA interference

HeLa cells were plated at a density of 2×10^5 cells per single well of a 6-well plate. The day after, cells were transfected in fresh media with 80 nM siRNA duplexes targeting either human PHD2 (5'-ggacgaaagccaugguugcuuguua-3', sense strand) or PHD3 (5'-gcuauccgggaaauggaacagguua-3' sense strand) using Lipofectamin 2000 according to the manufacturer's protocol (Invitrogen). After 4 hours of culturing at 20% oxygen, cells were subjected to the indicated

oxygen concentrations and grown for an additional period of 24 hours prior to lysis.

2.2.3.8 Expression and purification of PHDs

GST.PHD2 and GST.PHD3 were expressed in baculovirus-infected Sf9 insect cells according to the manufacturer's instructions (Invitrogen). After 80 hours of infection, Sf9 cells were lysed in ice-cold 0.1% NP-40, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 100 mM glycine and 10 μM DTT. Crude lysate was cleared by centrifugation at 20,000×g for 20 minutes and supernatants were incubated with equilibrated glutathione-sepharose beads (Amersham, Dübendorf, Switzerland) for 2 hours at 4°C with gentle agitation. Beads were washed three times with PBS and bound protein was eluted with 15 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0, 5% glycerol and 2 μM $FeSO_4$. Purity of recombinant proteins was estimated by SDS-PAGE and coomassie blue staining.

2.2.3.9 In vitro prolyl-4-hydroxylation assays

Enzymatic activity of recombinant PHD2 and PHD3 was determined by an *in vitro* hydroxylation assay performed essentially as described before (Oehme *et al.* 2004; Martin *et al.* 2005). Briefly, biotinylated mouse HIF-1α-derived peptides (aa 555 to 573, either wild-type or P563A mutant) were bound to NeutrAvidin-coated 96-well plates (Pierce). Hydroxylase reactions using purified recombinant GST.PHD2 or GST.PHD3 enzyme were carried out for 1 hour at room temperature. A polycistronic expression vector for His_6- and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex was kindly provided by S. Tan (Pennsylvania, PA). VBC was expressed in bacteria, purified by nickel affinity chromatography followed by ion exchange chromatography (Amersham), and allowed to bind to the hydroxylated peptides. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma) using the TMB (3,3',5,5'-tetramethylbenzidine) substrate kit (Pierce). The peroxidase reaction was stopped by adding H_2SO_4 and absorbance was determined at 450 nm in a microplate reader. For oxygen

titration experiments, the assay was performed in the hypoxic glove box. All reagents and solutions were allowed to equilibrate to the indicated oxygen concentration. Inter-assay comparability was guaranteed by calibration of each experiment to an internal standard curve using hydroxyproline-containing peptides.

2.2.4 Results

2.2.4.1 mRNA levels of inducible PHD isoforms show a transient maximum and remain upregulated during prolonged hypoxia in cell culture.

To study the kinetics of PHD expression during prolonged hypoxia (2% O_2 for up to 256 hours), mRNA levels of all three PHDs were determined in MEF-*Hif1a*$^{+/+}$ and MEF-*Hif1a*$^{-/-}$ cells. PHD2 and PHD3 but not PHD1 mRNA levels were efficiently upregulated under hypoxic conditions, peaked after 64 to 112 hours, and remained elevated over the entire time-course (Fig. 1A). As positive controls, mRNA levels of GLUT1 and CAIX, two well established HIF-1 target genes, were measured in the same time-course. Interestingly, hypoxic GLUT1 upregulation followed a similar kinetics as PHD2 mRNA expression, while CAIX levels were maximally induced 48 hours later, like it was observed for PHD3 (Fig. 1A). Because both PHDs were readily induced after four hours, we investigated the early onset of induction in a shorter time-course. Induction of PHD2 and PHD3 mRNA was detectable as early as 60 to 120 minutes after hypoxic stimulation (Fig. 1B). None of the PHD isoforms was induced in hypoxic MEF-*Hif1a*$^{-/-}$ cells, suggesting a non-redundant role of HIF-1α for hypoxic upregulation of PHD mRNA in these cell lines. In addition, a lack of PHD2 and PHD3 mRNA induction was also observed in functionally HIF-1β/ARNT-deficient mouse Hepa1c4 but not wild-type Hepa1 hepatoma cells after 38 hours of hypoxic stimulation (Fig. 1C). Thus, these genetically altered cellular models confirm HIF-1-dependent PHD2 and PHD3 gene expression in both acute and chronic hypoxia, which has previously been demonstrated mainly in siRNA experiments (Berra *et al.* 2003; Aprelikova *et al.* 2004; Marxsen *et al.* 2004).

2.2.4.2 Tissue-dependent hypoxic induction of PHD2 and PHD3 mRNA in mice.

Up to date, no systematic investigation on hypoxic PHD mRNA induction *in vivo* was available. Thus, we assessed the grade of hypoxic PHD2 and PHD3 mRNA induction in acutely and chronically hypoxic mice.

To examine the effects of acute hypoxia, mice were treated for 4 hours with an inspiratory gas-mixture containing 0.1% carbon monoxide (CO) which induced a rapid onset of severe hypoxia by blocking approximately 50% of the oxygen-binding sites in hemoglobin (data not shown). Strong hypoxic induction of erythropoietin mRNA content in kidney as well as erythropoietin and GLUT1 mRNA in liver and brain confirmed the activation of the HIF system in these mice (Fig. 2). The various PHD mRNA isoforms showed a broad disparity of hypoxic activation: PHD1 was not markedly regulated by hypoxia in most tissues; PHD2 was widely induced by hypoxia, albeit at rather low levels; and PHD3 was strongly induced already following four hours tissue hypoxia in the lung (15.6-fold), liver (5.2-fold), kidney (3.8-fold), but only moderate induction factors similar to PHD2 were observed in other organs. Of note, striated muscle tissue (heart and to some extent tongue) showed highest normoxic expression values for the inducible PHD2 and PHD3 isoforms.

To investigate the kinetics of PHD mRNA induction under chronically hypoxic conditions, mice were exposed to inspiratory hypoxia (7.5% O_2) for 24 up to 72 hours and mRNA levels were determined in liver, kidney and brain. While erythropoietin mRNA expression was induced to a similar extent in kidney and brain already after 24 hours of hypoxia, erythropoietin mRNA in liver increased not until 72 hours of hypoxic exposure (Fig. 3A). Consistent with previous findings, serum levels of erythropoietin protein in these animals highly correlated with mRNA levels in kidney (Fig. 3B). Unexpectedly, the hypoxic inducibility of the known HIF target genes GLUT1 and CAIX was rather small. While strong hypoxic induction of both proteins in tumors and tumor-derived cell lines is well established, they were not induced in liver, only one of them (CAIX) was induced after prolonged hypoxia in kidney and both of them showed a transient induction in brain (Fig. 3C). Nevertheless, these

results confirmed the hypoxic status of the animals. Comparable results were obtained with the inducible PHD2 and PHD3 isoforms: only PHD2 showed a small, transient increase in the liver; only PHD3 was induced after prolonged hypoxia in the kidney; and both of them were transiently induced in the brain. Thus, under physiological inspiratory hypoxia, regulation of these genes appears to be more subtile than under *in vitro* conditions, in tumor hypoxia or following severe tissue hypoxia in CO-treated mice.

2.2.4.3 Hypoxic induction of PHD2 and PHD3 proteins is accompanied by decreased HIF-1α protein levels.

Hypoxic upregulation of PHD2 and PHD3 has the potential to affect protein abundance of HIF-1α during prolonged hypoxia. To further elaborate this hypothesis, we quantified protein levels of HIF-1α as well as PHD2 and PHD3 in different cell lines. As shown exemplarily for HEK293 cells, endogenous HIF-1α protein levels decreased concomitantly with the hypoxic increase of endogenous PHD2 and PHD3 levels when cells were cultured for up to 72 hours at 1% O_2 (Fig. 4A). Similar results were obtained from lysates that were prepared under strictly hypoxic conditions, ruling out the possibility that increased levels of induced PHD enzymes might have influenced the protein levels of HIF-1α during lysis. HIF-1α mRNA levels in HEK293 were not significantly altered under the same hypoxic conditions, while PHD2 and PHD3 mRNAs were readily induced after 4 hours hypoxia (Fig. 4B). Interestingly, hypoxic PHD3 mRNA induction was steadily induced during the entire hypoxic incubation, whereas PHD2 mRNA levels reached maximal induction already before 4 hours of hypoxia and remained constant for up to 72 hours of hypoxia.

A similar pattern of HIF-1α and PHD protein levels was observed in HeLa and Hep3B cells at oxygen concentrations as low as 0.2%, supporting the idea of a widespread regulative mechanism (see Supplemental Fig. 1). These observations suggest that even under very limited oxygen supply HIF-1α hydroxylation by PHDs functionally persists, implicating that HIF-1α turn-over

might be controlled by the PHD sensor system not only under normoxic conditions but also under severely hypoxic conditions.

2.2.4.4 A second hypoxic insult activates another HIF-1α response in cells adapted to chronic hypoxia.

If the novel HIF-1α protein baseline following adaptation to chronic hypoxia indeed resulted from an altered steady-state of the HIF-PHD oxygen sensing circuit, the adapted cells should react to a second, more severe hypoxic insult by acutely inducing HIF-1α to a similar extent as at the first hypoxic insult. To test this hypothesis, HEK293 cells were allowed to adapt to 1% oxygen for 72 hours before they were exposed to 0.2% oxygen for up to 4 hours. While HIF-1α levels markedly decreased during chronic hypoxia, a rapid re-accumulation of HIF-1α protein was observed already one hour after exposing the cells to 0.2% oxygen (Fig. 4C). A similar result was obtained when cells adapted to chronic hypoxia were treated with the PHD inhibitor DMOG, suggesting that PHDs are responsible for HIF-1α regulation even in hypoxically adapted cells (Fig 4D).

2.2.4.5 PHD2 and PHD3 silencing increases hypoxic HIF-1α accumulation.

Like in HEK293 cells, simultaneous treatment of HeLa cells with hypoxia together with the PHD inhibitor DMOG led to additional accumulation of HIF-1α at oxygen concentrations as low as 0.2% (Fig. 5A). To provide further evidence that endogenous PHDs control HIF-1α protein levels even under hypoxic conditions, we applied siRNA to knock down PHD2 and PHD3 in HeLa cells. Interestingly, silencing of either PHD2 or PHD3 equally increased HIF-1αlevels, irrespective of whether the cells were cultured at 20% or 1% oxygen (Fig. 5B). While apparently both isoforms are involved in regulating HIF-α stability over a broad range of oxygen concentrations, combined silencing of PHD2 together with PHD3 most efficiently upregulated HIF-1α at 20% as well as 1% O_2 (Fig. 5B).

2.2.4.6 PHDs retain functional HIF-1α degradation activity even under severely hypoxic conditions in cell culture.

The current knowledge about oxygen substrate requirements of the PHD-dependent hydroxylation reaction is exclusively based on data derived from biochemical *in vitro* studies using purified enzymes and short peptides containing one single HIF-1α hydroxylation site as substrates. To further examine the activity of PHD enzymes under lowered oxygen availability in a cellular context, a luciferase-based mammalian one-hybrid approach to quantify HIF-1α stability in a feedback-uncoupled cellular system was established. A series of mammalian expression vectors coding for the mouse HIF-1α ODD domain, including mutant forms of the hydroxylation sites (P402A and/or P563A) were N-terminally fused to the yeast GAL4 DNA-binding domain (DBD) and C-terminally fused to the *herpes simplex* virus VP16 transactivation domain (TAD; Fig. 6A). The expressed fusion proteins cannot transactivate endogenous HIF-target genes, including PHD2 and PHD3, but activate a co-transfected GAL4-responsive reporter gene. The use of HIF-1α-deficient MEF-*Hif1a*$^{-/-}$ cells ensures very low basal levels of endogenous PHDs (Fig. 1A) and avoids confusion with hypoxic upregulation of endogenous PHD2 and PHD3 by endogenous HIF-1, allowing to experimentally define the PHD levels by transfection of expression vectors. Since the HIF-1α-ODD confers oxygen-dependent instability to the fusion protein, luciferase reporter gene activity directly reflects protein stability.

First, we tested the relative importance of the two PHD target proline residues within the mouse HIF-1α-ODD residues 359 to 685. While reporter gene activation of the wild-type and single proline mutant fusion proteins was similarly reduced by PHD2 overexpression, mutation of both prolines was necessary to render the HIF-1α-ODD insensitive to PHD2 overexpression (Fig. 6B). Whereas similar effects of PHD overexpression on ODD-stability have previously been reported only for normoxic conditions (Masson *et al.* 2001), our data show functional relevance of both hydroxylation sites also for hypoxic (2% O_2) regulation of HIF-1α protein levels (Fig. 6B).

In a next step both, oxygen concentration and PHD protein abundance were titered simultaneously in co-transfection experiments. Interestingly, co-expression of low amounts of PHD2, which were not yet detectable by immunoblotting, already reduced HIF-1α-ODD stability to a minimum which was not further lowered by increased amounts of PHD2 (Fig. 6C). Reducing the oxygen concentration to 4% or below revealed a successive decrease of hydroxylase activity at a given amount of PHD2 enzyme. However, increasing the abundance of PHD2 by stepwise duplication of the amount of transfected PHD2 expression vector, again demonstrated that induced PHD levels can compensate for reduced oxygen availability. Indeed, increased PHD2 compensated for oxygen concentrations as low as 0.2% (Fig. 6C).

Similar results were obtained by PHD3 overexpression which also resulted in decreased HIF-1α-ODD stability under normoxic and moderately hypoxic conditions (Fig. 6D). However, forced expression of PHD3 failed to further decrease HIF-1α-ODD stability at 0.2% oxygen, suggesting a principal difference in oxygen-dependent hydroxylase activities between PHD2 and PHD3 under various degrees of hypoxia *in vivo* (Fig. 6D).

2.2.4.7 Purified PHDs retain functional hydroxylation activity even under severe hypoxic conditions in a cell-free system in vitro

The differences in hydroxylase activity of PHD2 and PHD3 under severe hypoxia described above prompted us to investigate the activity of PHDs using a cell-free *in vitro* approach. Therefore, GST-tagged PHD2 and PHD3 were purified from baculovirus-infected Sf9 insect cells (Fig. 7A). These enzymes were used to hydroxylate a mouse HIF-1α-ODD-derived peptide coupled to 96-well plates, and binding of a purified VBC complex to the hydroxylated peptide was measured by ELISA. Each PHD preparation was diluted to obtain equal VBC-binding after one hour of hydroxylation (arbitrarily defined as “1-fold input”). These enzyme concentrations were subsequently increased to mimic the hypoxic induction of PHDs *in vivo*. Under normoxic conditions, both PHDs showed a similar increase in activity with increasing protein amounts (Fig 7A). Peptide hydroxylation was within the linear range of

the assay over the time frame of experiments, even at highest enzyme concentrations (data not shown).

When the assay was performed at an oxygen concentration of 2%, PHD2 hydroxylation activity was only about half of the normoxic activity after one hour of reaction (hydroxyproline contents of 10.1% ± 0.2% at 20% O_2 and 5.7% ± 0.5% at 2% O_2; n=3, mean ± S.D.; 8-fold enzyme input). However, HIF-1α-ODD peptide hydroxylation by PHD3 was only slightly decreased under these conditions (10.3% ± 0.2% at 20% O_2 and 8.2% ± 1.1% at 2% O_2; n=3, mean ± S.D.; 8-fold enzyme input). Further reduction in oxygen availability decreased proline hydroxylation, but significant hydroxylation could still be observed at oxygen concentrations as low as 0.2%. Indeed, a 8-fold protein input of PHD2 or PHD3 resulted in similar peptide hydroxylation at 0.2% O_2 as a 4-fold input at 0.5% O_2 or a 2-fold input at 1% O_2, suggesting that increased PHDs can compensate for decreased oxygen also *in vitro* (Fig 7A). Moreover, both enzymes showed a strikingly linear increase of activity for the range of 0.2% to 2% oxygen, thus fulfilling one of the most important criterions for a cellular oxygen sensor at physiologically relevant tissue O_2 concentrations (Fig. 7B).

2.2.5 Discussion

The existence of a functional feedback loop limiting the hypoxic response has been suspected already when HIF was cloned, because nuclear HIF-1α declined despite ongoing hypoxia (Wang *et al.* 1995). Similar observations were then reported by a multitude of other groups. However, the underlying mechanism remained unknown. We previously reported that forced expression of HIF-1α followed a similar feedback kinetics, but in this case under normally oxygenated conditions (Hofer *et al.* 2001). This observation suggested that HIF itself, rather than the hypoxic stimulus, is required for the feedback regulation. One such mechanism might be HIF-dependent induction of pVHL by "late hypoxia" (Karhausen *et al.* 2005). However, other studies could not find any induction of pVHL under hypoxic conditions (Berra *et al.* 2001; Demidenko *et al.* 2005), and we did not observe any oxygen- or HIF-

1α-dependent difference in pVHL mRNA levels during the 256 hours time-course described in this study (data not shown).

The identification of the PHD oxygen sensors shed new light on the process of HIF feedback control, as it became clear that PHD2 and PHD3 are hypoxia-inducible HIF target genes. (Epstein *et al.* 2001; D'Angelo *et al.* 2003; Appelhoff *et al.* 2004; Aprelikova *et al.* 2004; Marxsen *et al.* 2004; Metzen *et al.* 2005; Pescador *et al.* 2005). It has been postulated that this PHD upregulation confers accelerated degradation of HIFα upon re-oxygenation (Berra *et al.* 2001; Appelhoff *et al.* 2004; Marxsen *et al.* 2004). Indeed, re-oxygenation from 1% to 20.9% (air) oxygen after 18 hours decreased the half-life of HIF-1α when compared with similarly treated cells that were exposed to hypoxia for only one hour (Marxsen *et al.* 2004).

However, physiological tissue pO_2 corresponds to approx. 2% to 5% oxygen concentrations in air rather than the widely used 20.9% "normoxic" oxygen concentration. Even though the pericellular pO_2 is lower at the bottom of unstirred tissue culture dishes (Wolff *et al.* 1993), standard cell culture conditions are to be considered “hyperoxic”; which reflects an unphysiological condition PHDs normally are not exposed to. Rather, PHDs *in vivo* are constantly functioning under pO_2 levels far below their *in vitro* K_m values (Hirsila *et al.* 2003). These considerations suggested a PHD-HIF feedback loop which might be active even under chronically low tissue oxygenation.

In line with this hypothesis, we observed undulating HIF-1α-dependent mRNA levels of CAIX, GLUT1, PHD2 and PHD3 in MEF cells, and we detected tissue-specific variations in hypoxically induced PHD2 and PHD3 mRNA levels in mice *in vivo*. So far PHD expression levels were only known from established tumor cell lines or normoxic tissues (Dupuy *et al.* 2000; Lieb *et al.* 2002; Oehme *et al.* 2002; Berra *et al.* 2003; Appelhoff *et al.* 2004; Metzen *et al.* 2005). We found an impressive upregulation of PHD3 mRNA levels in hypoxic lung tissue, confirming previous reports for lung cancer-derived A549 cells (Appelhoff *et al.* 2004; Demidenko *et al.* 2005). Similarly, the observed induction factors in liver were roughly the same as determined in Hepa1 hepatoma cells used in this study.

We then concentrated on three major organ systems which are particularly sensitive to hypoxia and hence involved in the pathophysiology of shock. Interestingly, while the examined HIF target genes were all induced in the brain, different kinetics for GLUT1, CAIX, PHD2 and PHD3 were observed in kidney and liver. Expression levels of CAIX were highly increased in kidney after 72 hours of hypoxia, whereas a reverse effect was observed in liver. Since the kidney, besides the lung, is the major organ involved in systemic pH regulation, these results suggest a physiological role for CAIX in pH maintenance during hypoxia, like it has been proposed for solid tumors (Svastova *et al.* 2004). Regarding PHD mRNA induction, temporal upregulation of at least one isoform was observed in each of the three tissues in hypoxic animals, confirming that PHD upregulation indeed occurs in a hypoxic organism, albeit the extent of induction is tissue-specific. Not surprinsingly, decreasing the oxygen transport capacity in mice by 0.1% CO inspriation was much more efficient in upregulation of HIF-target genes than inspiratory hypoxia itself, suggesting that adaptational mechanisms, such as increased heart rate, depth of breath, and reduced oxygen consumption compensated for decreased inspiratory O_2-concentration when oxygen transport is intact. We have previously shown that temporal and spatial accumulation of HIF-1α *in vivo* greatly differs, even in neighboring cells, suggesting that factors other than oxygen availability affect the PHD-HIF system (Stroka *et al.* 2001). In fact, we recently found that the FK506-binding protein FKBP38 regulates specifically the abundance of PHD2 (S. Barth, R. Wirthner, R. H. Wenger and G. Camenisch, manuscript in preparation).

Whatever regulates cellular PHD levels, functional PHD induction requires that these enzymes must be active under a broad range of physiologically relevant conditions. In order to experimentally support this hypothesis, we used a HIF-1α-ODD reporter construct in a HIF-1α negative cell line to breach the suggested gene activation-protein degradation loop. The HIF-1α-ODD confers normoxic degradation to the resulting hybrid fusion protein (Pugh *et al.* 1997) and similar constructs have been used lately as bioluminescent reporter systems monitoring the state of tissue-oxygenation *in*

vivo (Safran *et al.* 2006). Our experiments revealed that PHD dependent fusion-protein destabilization was effective at oxygen concentrations as low as 0.2% O_2, and that increasing the amount of enzyme partially compensated for reduced oxygen availability. These data are consistent with a recent work employing hydroxylation-specific antibodies which showed persistent HIF-1α hydroxylation at equally low oxygen concentrations (Chan *et al.* 2005).

While we observed a comparable oxygen-dependence between PHD2 and PHD3 purified from Sf9 insect cells *in vitro*, the ability of PHD3 to compensate for low oxygen concentrations in the cellular model was consistently lower than that of PHD2 (compare Figures 6C and D). In line with the former observation, *in vitro* transcribed and translated PHD2 and PHD3 enzymes have been shown to be equally active in another study (Tuckerman *et al.* 2004). Thus, isoform-specific regulation of distinct PHDs *in vivo* are likely to depend on critical co-factors that might be depleted in purified enzyme preparations, or becoming limited when PHDs are overexpressed. Supporting this notion, PHD3 has recently been described to serve as a substrate of the eukaryotic chaperonin complex TRiC that might be required for appropriate folding and full enzymatic activity(Masson *et al.* 2004).

Another recent work demonstrated increased binding of PHD3 to HIF-1α in the presence of OS-9 (Baek *et al.* 2005). As suggested by these authors, PHDs form functional multi-protein complexes which could affect binding affinity and target sequence selectivity in a cellular context. However, binding of OS-9 to human HIF-1α necessarily requires amino acids 692–785 which were neither present in the peptide used for the hydroxylation assays nor in the one-hybrid construct. Thus, PHD3 activity rather than binding might be attenuated in our cellular model system. Of note, protein degradation of PHD3 (and PHD1) has been shown to be enhanced under hypoxia by a mechanism involving the ubiquitin ligase Siah2, adding yet another layer of regulation on the HIF-PHD system (Nakayama *et al.* 2004).

Also, binding of PHD2 to HIF-1α has been suggested to repress the N-TAD activity (To and Huang 2005). Interestingly, we observed residual binding of recombinant PHD2 to immobilized HIF-1α ODD peptides *in vitro*,

suggesting a repressive function of PHD2 particularly on truncated HIF-1α (data not shown). While these effects were rather small compared with the HIF-1α-destabilizing function of enzymatic PHD activity, they still could have partially influenced the transcriptional activity of the fusion constructs used in our study.

In conclusion, the present work demonstrates that an essential pre-requirement for a PHD abundance-dependent modulation of the oxygen-sensing system is fulfilled: PHDs are operative under a wide variety of even severely hypoxic oxygen concentrations. These findings led to the intriguing suggestion of a flexible oxygen threshold of the PHD-HIF system which steadily adapts to altered tissue oxygenation.

2.2.6 Acknowledgments

The authors wish to thank R. S. Johnson, L. Poellinger, W. Kaelin Jr., I. Flamme, D. Peet, P. J. Ratcliffe, Z. A. Ronai and S. Tan for providing cells, antibodies and plasmids, and B. Kuppusamy for helpful discussion.

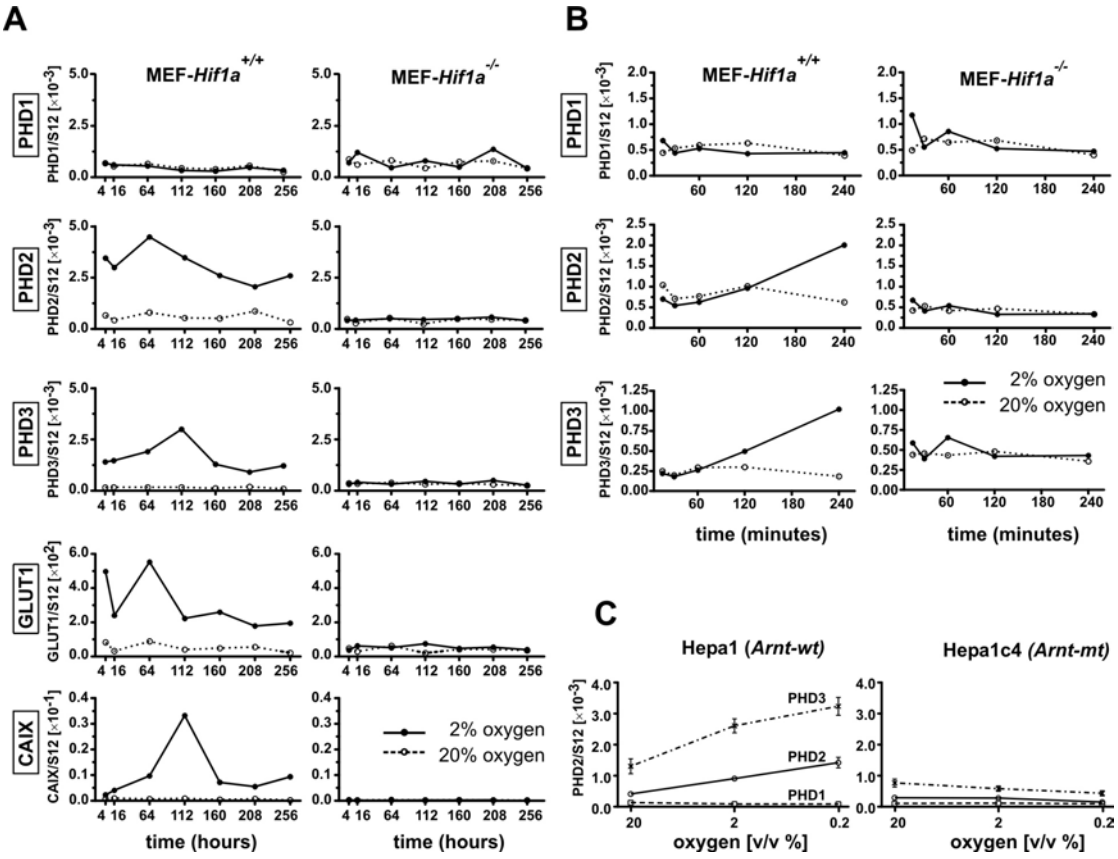

Figure 1. HIF-1 target genes remain elevated during prolonged hypoxia. MEF-*Hif1a*$^{+/+}$ and MEF-*Hif1a*$^{-/-}$ were cultured either at 20% or 2% oxygen for 4 to 256 hours (A) or 15 to 240 minutes (B). mRNA levels for PHD1, PHD2, PHD3, GLUT1 and CAIX were quantified by real-time RT-PCR and normalized to ribosomal protein S12 mRNA content. (C) PHD1, PHD2 and PHD3 mRNA levels in Hepa1 Arnt wild-type (wt) and Hepa1c4 Arnt mutant (mt) cells cultured at 20%, 2% or 0.2% oxygen for 38 hours. Data are given as mean ± SEM of n = 3 independent experiments.

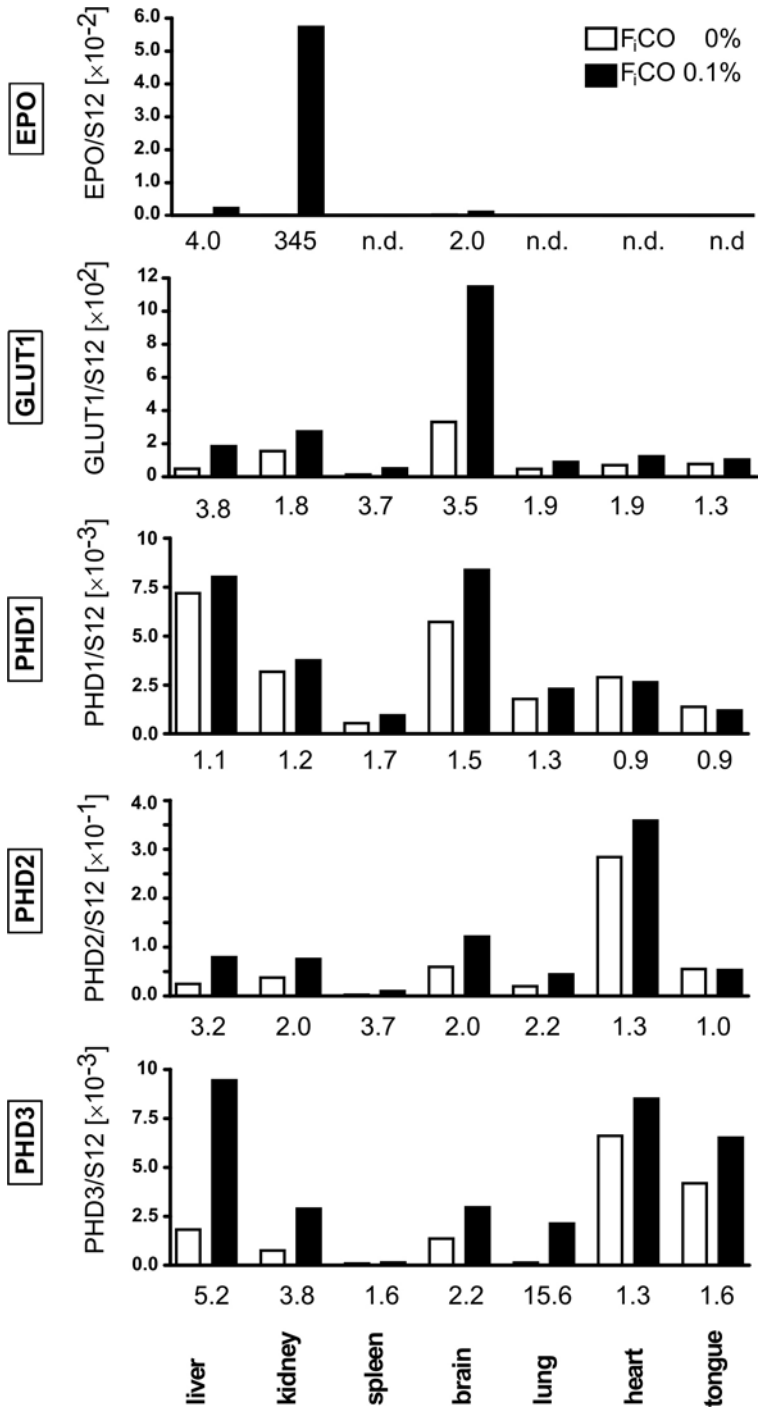

Figure 2. Effects of tissue hypoxia on the inducibility of PHD isoforms *in vivo*. Tissues were derived from mice kept either under normal air (FiCO 0%) or in a gas mixture containing 0.1% CO (FiCO 0.1%) for 4 hours. PHD1, PHD2, PHD3, erythropoietin and GLUT1 mRNA levels were determined by real-time RT-PCR and normalized to S12 mRNA. Organ-specific hypoxic induction factors are indicated below the bars. Note the different scales (n.d., not detectable).

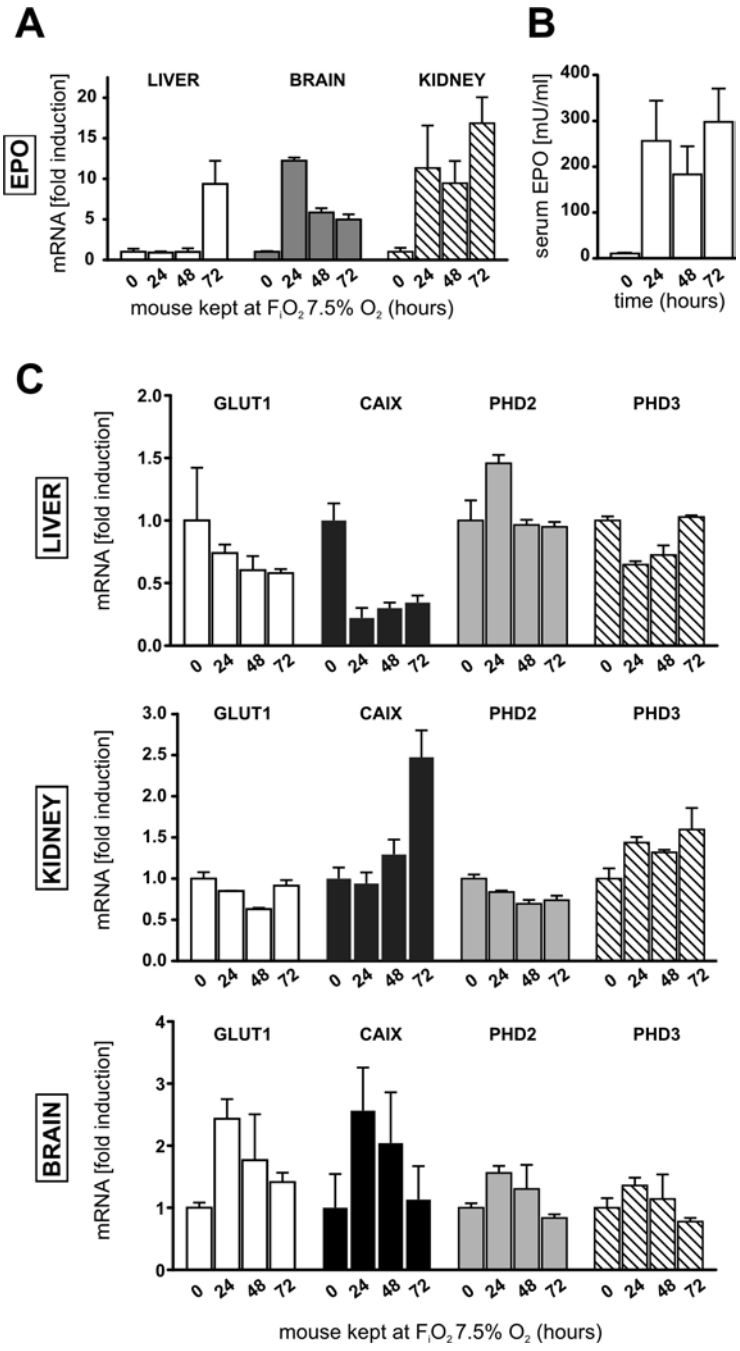

Figure 3. Effects of inspiratory hypoxia on HIF target gene expression *in vivo*. Mice were kept at an inspiratory oxygen concentration (FiO2) of 20% O2 ("0" hours of hypoxia) or 7.5% O2 for up to 72 hours. erythropoietin mRNA levels in liver, brain and kidney were quantified by real-time RT-PCR (A) and corresponding serum levels of erythropoietin were measured by RIA (B). (C) GLUT1, CAIX, PHD2 and PHD3 mRNA levels were measured in all three tissues. All mRNA data were normalized to S12 mRNA levels and are expressed as hypoxic induction factors. Bars represent the mean ± SEM of n = 3 mice per group.

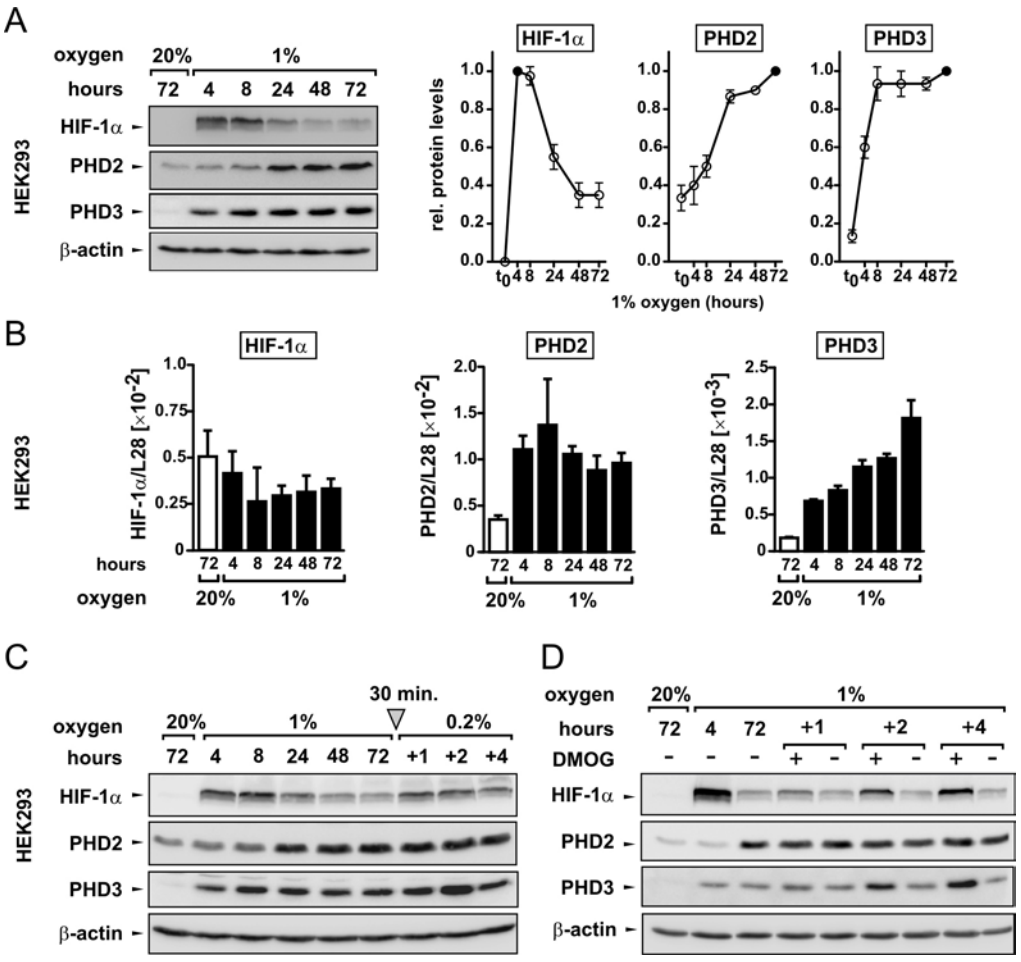

Figure 4. Decreased HIF-1α protein levels parallel increased PHD2 and PHD3 under chronically hypoxic conditions. (A) HEK293 cells were cultured at 20% or 1% oxygen for up to 72 hours. HIF-1α, PHD2, PHD3 and β-actin (loading control) protein levels were estimated by immunoblotting of total cell lysates. A representative experiment is shown along with densitometric quantification of bands for n = 3 (PHDs) or n = 4 (HIF-1α) independent experiments. For inter-assay normalization, timepoints expressing highest protein levels were arbitrarily defined as 1 (filled circles; mean ± SEM). (B) HIF-1α, PHD2 and PHD3 mRNA levels in HEK293 cells were quantified by real-time RT-PCR and normalized to ribosomal protein L28 mRNA. Bars represent mean ± SEM of triplicates. (C) HEK293 cells were exposed to 1% oxygen for 72 hours and subjected to 0.2% oxygen for additional 1 to 4 hours without reoxygenation. Lowering the oxygen concentration from 1 to 0.2% took approx. 30 minutes. Immunoblotting was performed as in (A). (D) HEK293 cells were grown as in (C) and treated with a PHD inhibitor ("+", 1 mM DMOG) or solvent alone ("-", 0.1% DMSO) for 1 to 4 hours at 1% O2. Immunoblotting was performed as described in (A).

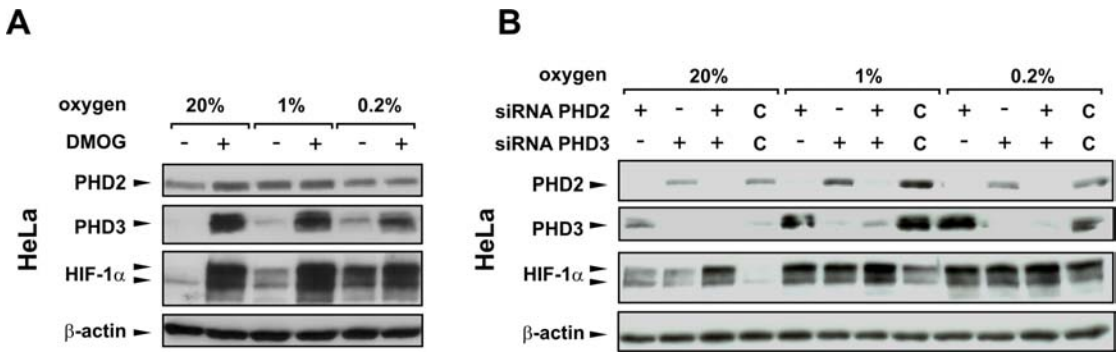

Figure 5. Endogenous PHD2 and PHD3 enzymes control HIF-1α accumulation under normoxic as well as under hypoxic conditions. (A) HeLa cultures were subjected to 20%, 1% or 0.2% oxygen for 4 hours and simultaneously treated with a PHD inhibitor ("+", 1mM DMOG). PHD2, PHD3, HIF-1α and β-actin protein levels were determined by immunoblotting of total cell lysates. (B) HeLa cells were transiently transfected with siRNAs directed against either PHD2, PHD3, or a combination of both, and cultured at the indicated oxygen concentrations for 24 hours. Transfection of cells with a similarly constructed, but unspecific siRNA oligonucleotide served as negative control ("C"). Immunoblotting was performed as described above.

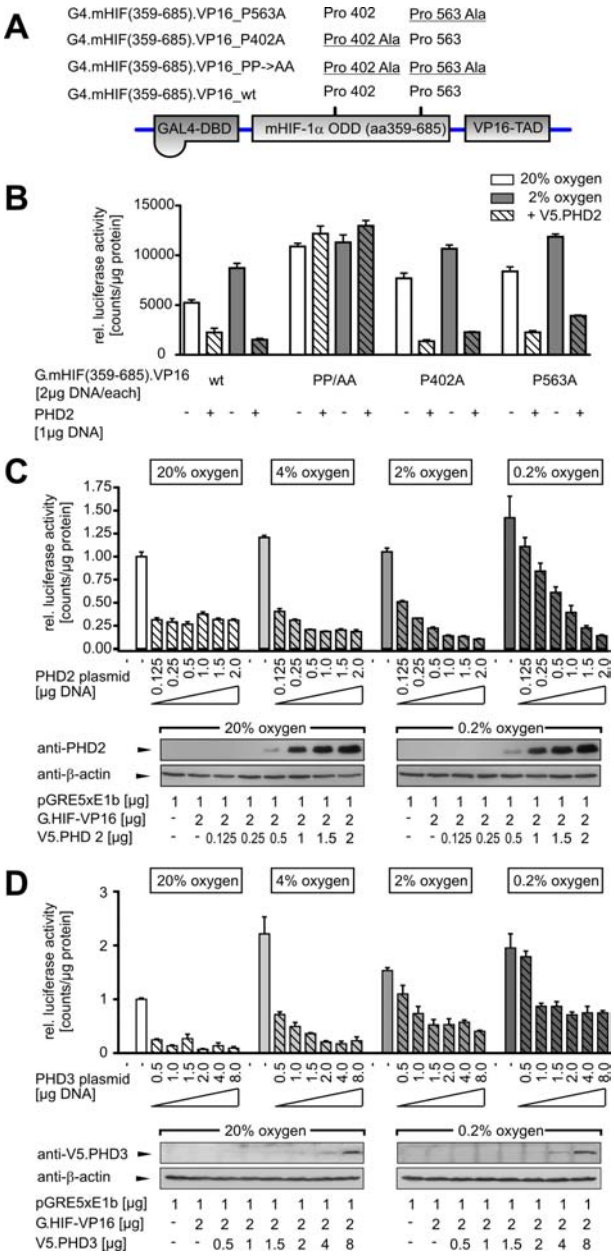

Figure 6. PHD2 and PHD3 retain hydroxylase activity at low oxygen concentrations. (A) Oxygen-sensitive one-hybrid fusion constructs. Mutations of Pro402 and Pro563 are underlined. (B) MEF-*Hif1a*$^{-/-}$ cells were co-transfected with 2 µg one-hybrid construct, 1 µg of a GAL4-responsive reporter gene and 1 µg of V5.PHD2 or empty expression vector. Luciferase activity was measured 24 hours after incubation at 20% or 2% oxygen and relative light counts were normalized to total protein content in the lysates. Likewise, the indicated amounts of V5.PHD2 (C) and V5.PHD3 (D) were co-transfected with 2 µg of the wild-type one-hybrid construct and 1 µg of the reporter gene. Aliquots of transfected cells were then cultured simultaneously at various oxygen concentrations and luciferase activities determined after 24 hours as described above. In addition, lysates were immunoblotted to verify expression of exogenous V5.PHD isoforms (bottom panels). Representative experiments performed in triplicates are shown as mean ± SEM.

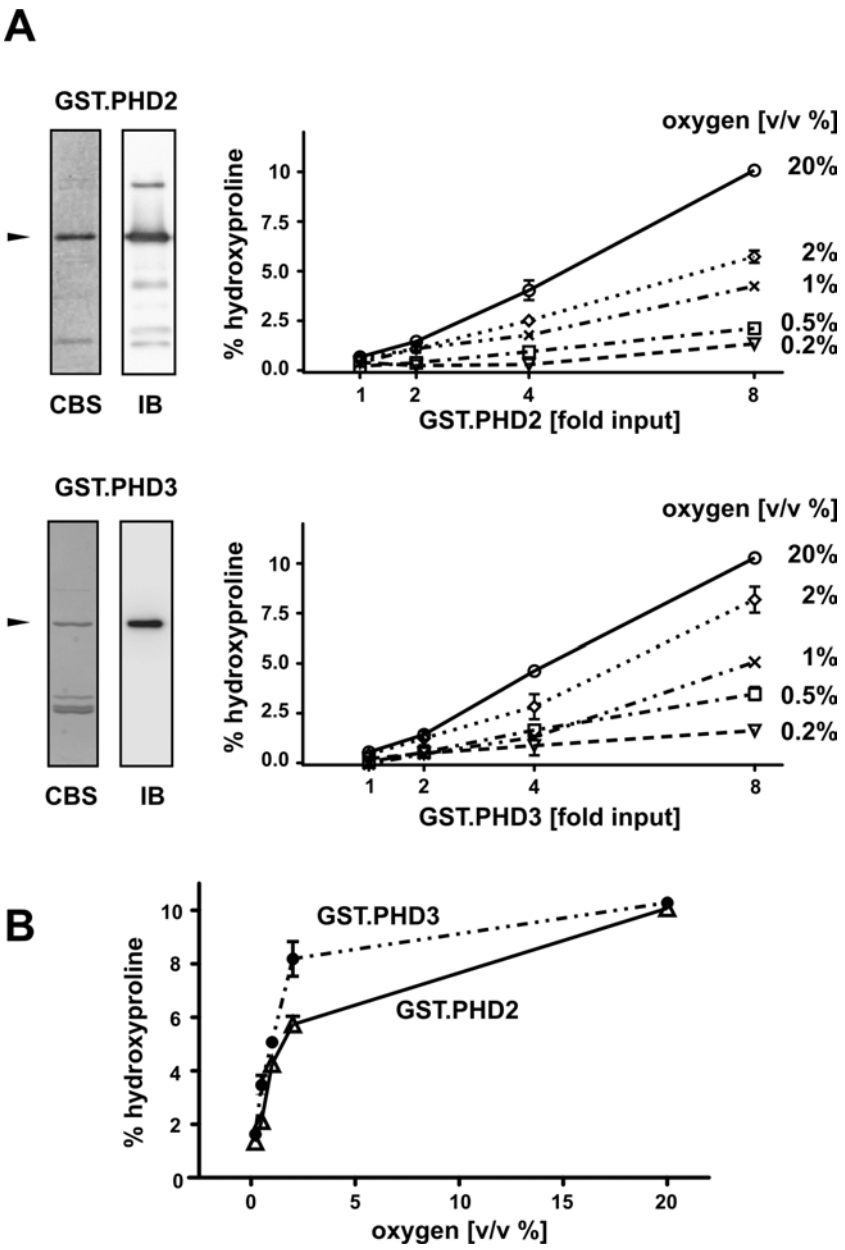

Figure 7. Recombinant GST.PHD2 and GST.PHD3 proteins are active under hypoxic conditions *in vitro*. (A) GST.PHD2 and GST.PHD3 were purified from baculovirus-infected Sf9 insect cells and their purity checked by SDS-PAGE and coomassie blue staining (CBS) or by immunoblotting (IB) using polyclonal anti-PHD2 or anti-PHD3 antibodies. In vitro hydroxylation reactions were performed at various oxygen concentrations with increasing amounts of PHD2 and PHD3 enzymes and hydroxyproline content was estimated from a calibration curve. *(B)* Oxygen-dependency of hydroxylation is shown for 8-fold enzyme input. Enzyme activity is given as percentage of hydroxyproline after one hour of reaction. All values are in the linear range of the assay and are shown as mean values ± SEM of triplicates.

2.3 Hypoxia-inducible factor-dependent DNA double-strand break repair contributes to tumor cell chemoresistance

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2.3.1 Abstract

A mismatch between metabolic demand and nutrient delivery leads to microenvironmental changes in solid tumors. The resulting tumor hypoxia is associated with malignant progression and poor prognosis. Clinically, tumor hypoxia is also associated with resistance to chemotherapy and irradiation. However, the molecular mechanisms underlying therapy resistance in hypoxic tumors are not fully understood. The hypoxia-inducible factor (HIF) is a master transcriptional activator of oxygen-regulated gene expression. Using transformed mouse embryonic fibroblasts (MEFs) derived from mice deficient in HIF-1α, we previously found that the lack of HIF-1α is sufficient to confer increased chemotherapy and irradiation susceptibility. Here, we show by single-cell electrophoresis, histone 2AX phosphorylation and nuclear foci formation of γH2AX and 53BP1, that the number of DNA double strand-breaks (DSB) is increased in untreated and etoposide-treated HIF-deficient MEFs. In etoposide-treated cells, cell cycle control and p53-dependent gene expression were not affected by the absence of HIF-1α. Using a candidate gene approach to screen 17 genes involved in DNA repair, mRNA and protein of members of the DNA-dependent protein kinase (DNA-PK) complex were found to be decreased in HIF-deficient MEFs. Thus, these data establish a molecular link between HIF and DNA-DSB repair which is likely to contribute to HIF-dependent tumor therapy resistance.

2.3.2 Introduction

Due to inadequate vascularization, irregular blood flow, high oxygen consumption and sometimes anemia, solid tumors build up hypoxic areas, resulting in distinct tumor cell phenotypes. Tumor hypoxia is associated with malignant progression, increased metastasis, genetic instability, chemoresistance, radioresistance, and poor prognosis (Brown and Giaccia 1998; Brown 2000; Pouyssegur *et al.* 2006). Therefore, understanding the molecular pathways induced by hypoxia in tumor cells became a major focus in the development of new strategies to improve cancer therapy efficiency.

Central to the physiological response to hypoxia is the oxygen-labile α subunit of the hypoxia-inducible factor-1 (HIF-1) (Wenger 2002). HIF-1 activates a large number of oxygen-regulated genes required for the adaptation of normal cells to hypoxia (Wenger *et al.* 2005). In cancer, HIF-1 is responsible for vascular endothelial growth factor-dependent tumor angiogenesis, for carbonic anhydrase (CA) IX-dependent pH regulation, and for the increased glycolytic capacity of cancer cells, known as Warburg effect (Minchenko *et al.* 2002; Svastova *et al.* 2004). Thus, HIF-1 mediates many of the adverse effects of tumor hypoxia and is an established positive factor for tumor growth (Ryan *et al.* 1998; Ryan *et al.* 2000). Apart from tumor hypoxia stabilizing the HIF-1α protein, HIF-1 can be upregulated constitutively due to the loss of tumor suppressors such as pVHL, PTEN and p53, or oncogenes such as v-*src* (Brown and Giaccia 1998; Wenger 2002; Brown and Wilson 2004; Pouyssegur *et al.* 2006). Indeed, HIF is overexpressed in the majority of human cancers with expression levels correlating with malignancy and negative survival prognosis (Zhong *et al.* 1999; Birner *et al.* 2000; Aebersold *et al.* 2001).

High HIF-1α expression levels in human cancers are also associated with incomplete responses to chemotherapy and radiotherapy (Aebersold *et al.* 2001; Koukourakis *et al.* 2002; Bachtiary *et al.* 2003; Generali *et al.* 2006). However, the precise molecular mechanisms underlying hypoxic cancer therapy resistance are not fully understood. One of the first reported molecular mechanisms explaining the involvement of HIF-1 in chemotherapy resistance was the observation that HIF-1 can augment multidrug resistance (*MDR1*)

gene expression (Comerford *et al.* 2002). However, MDR1 is not induced in all therapy resistant cancers and is not involved in hypoxic radioresistance. Dysregulated apoptosis in response to chemotherapy might be another explanation (Erler *et al.* 2004)(17), but the cell type-specific role for HIF-1 in apoptosis is not well established because cells do not undergo apoptosis under degrees of hypoxia sufficient for HIF-1 induction (Wenger *et al.* 1998).

Resistance to radiotherapy is generally attributed to lowered generation of oxygen-dependent radical formation, at least under severe hypoxic conditions, below the oxygen partial pressure required to induce HIF-1 (Moeller and Dewhirst 2006). However, we previously reported that transformed mouse embryonic fibroblasts (MEFs) were more sensitive to chemotherapy as well as to radiotherapy in the absence of HIF-1α (Unruh *et al.* 2003). Experimental tumors using these cells were also more susceptible to chemotherapy when HIF-1α was absent. These data were confirmed by a large number of studies that showed reversal of chemoresistance as well as radioresistance by targeting HIF-1 (Zhang *et al.* 2004; Moeller *et al.* 2005; Williams *et al.* 2005; Brown *et al.* 2006; Li *et al.* 2006; Li *et al.* 2006; Song *et al.* 2006; Sasabe *et al.* 2007). On the other hand, experimentally increasing HIF-1α enhanced therapy resistance (Ji *et al.* 2006; Martinive *et al.* 2006). Because HIF-1-mediated therapy resistance was only observed when DNA double-strand break (DSB) but not single-strand break (SSB)-inducing treatments were applied, we suspected that HIF-1 might be involved in DNA-DSB repair (Unruh *et al.* 2003). Therefore, we further characterized this putative novel role of HIF-1 in DNA damage and repair.

2.3.3 Materials and Methods

2.3.3.1 Cell culture.

MEF-*Hif1a*$^{+/+}$ and MEF-*Hif1a*$^{-/-}$ cell lines were derived from day 9.5 embryos, either wild-type (*Hif1a*$^{+/+}$) or deficient (*Hif1a*$^{-/-}$) for HIF-1α. MEF cells obtained from independent mouse strains were either transformed with H-*ras* and immortalized with SV40 large T antigen (Ryan *et al.* 1998), herein after referred to as *r*T (kindly provided by R. S. Johnson, LaJolla, CA), or immortalized with SV40 large T antigen alone (Feldser *et al.* 1999), referred to

as T (kindly provided by G. L. Semenza, Baltimore, MD). All cell lines were cultured in Dulbecco's modified Eagle's medium (high glucose) as described previously (Stiehl *et al.* 2006). For long-term hypoxia (up to 256 hours), MEFs were grown in a gas-controlled glove box to handle the cells under constant oxygen (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Cells were grown on 145 mm culture dishes and splitted every 48 hours. Reagents used for splitting and permanent culturing were pre-equilibrated to the oxygen concentration in the glove box. Viability assays were performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described previously (Unruh *et al.* 2003).

2.3.3.2 Clonogenic assays.

Cells (1×10^6) were cultured on 150 mm tissue culture plates for 24 hours and subsequently treated with 1, 2.5 or 5 μM etoposide (Sigma, Buchs, Switzerland) for 60 minutes. Cells were then trypsinized and counted using a ViCell cell viability analyzer (Beckman-Coulter, Krefeld, Gemany). The cells (500 to 1,000) were seeded in triplicates on 100 mm culture plates and grown for an additional 6 days. Following fixation with 2% paraformaldeyde in PBS, the colonies were stained with crystal violet, counted and the numbers normalized to the untreated control groups.

2.3.3.3 Indirect Immunofluorescence.

MEFs (5×10^4 cells) were seeded on 24-well Lumox plates (Greiner, Frickenhausen, Germany) and treated with etoposide for 1 hour. Cells were rinsed with PBS and fixed with 2% paraformaldehyde in PBS for 5 minutes at room temperature, followed by permeabilization with 100% methanol at -30°C for 5 minutes. After blocking with 3% BSA for 1 hour, the primary antibodies rabbit anti-53BP1 (Novus Biologicals, Littleton, CO) and mouse anti-γH2AX (JBW103, Upstate, Charlottesville, VA) were allowed to bind for 2 hours and immunocomplexes were detected using secondary anti-rabbit Alexa488- and anti-mouse Alexa564-conjugates, respectively (Molecular Probes, Invitrogen, Basel, Switzerland). Nuclei were stained with DAPI. Membranes were cut, mounted in MOWIOL and dried over night in the dark. Epifluorescence was

analysed using an Axiovert fluorescence microscope (Zeiss, Feldbach, Switzerland) and images were captured with fixed exposure times.

2.3.3.4 Single cell electrophoresis (comet assays).

Single cell electrophoresis was performed essentially as described with minor modifications (33). Briefly, cells were grown on 100 mm plates and subjected to the indicated treatments. After rinsing with PBS, cells were detached using a 0.005% trypsin solution, collected by centrifugation at 4°C with 250×g and washed twice with ice-cold PBS. 2.5×10^4 cells were resuspended in 10 μl PBS and added to 190 μl of 0.5% low melting-point agarose (Sigma) equilibrated to 37°C in a water bath. Remaining cells were immediately lysed with 1% SDS, 50 mM Tris-HCl pH 6.8 and 10% glycerol for later immunoblot analyses. The agarose-cell suspension was subsequently distributed on dried, agarose-precoated fully frosted slides, covered with a cover slip and allowed to solidify on a precooled aluminium plate. Slides were overlaid with a third layer of agarose and then subjected to precooled lysis solution (1% Triton-X100, 2.5 M NaCl; 100 mM EDTA; 10 mM Tris-HCl pH 10.0) for 1 hour at 4°C in the dark. Afterwards, slides were equilibrated to alkaline electrophoresis buffer (300 mM NaOH; 1 mM EDTA) for 20 minutes to allow for unwinding of the DNA. The agarose-embedded cells were then subjected to horizontal electrophoresis for 30 minutes (~0.74V/cm; 300 mA) with corresponding slides running side by side to ensure equal electrophoresis conditions. Slides were neutralized for 20 minutes in 100 mM Tris-HCl pH 7.5 and allowed to dry over night in the dark. Before analysis, dried slides were reconstituted in water and stained with a SYBR green solution (Invitrogen) for 10 minutes. DNA migration was visualized by fluorescence microscopy and images captured using a fluorescence microscope. For quantitative comparism, the tail moment (%DNA in tail multiplied by tail length) was calculated from >150 cells per condition by two individuals using the CometScore software package (TriTek, Sumerduck, VA).

2.3.3.5 Cell cycle analyses.

MEFs were treated with 1 μM etoposide for 1 hour before the medium was replaced and the cells were cultured for an additional period of up to 48 hours.

At the indicated time points, cell samples were washed and trypsinized. Detached cells were fixed with precooled 70% ethanol at -30°C and the DNA was stained with propidium iodide (PI). FACS analysis was perfomed using a FACScan (Beckman-Coulter) and a single cell population of 25.000 cells was monitored for DNA content. Percentages of cells in G1, S phase and G2/M were estimated using WinMDI 2.9 freeware.

2.3.3.6 Protein extractions and immunoblot analyses.

Cells were washed twice and scraped into ice-cold PBS. For detection of DNA-PKcs, pATM, pCHK1, pCHK2, nuclei were extracted with a high-salt extraction buffer containing 0.1% NP-40 essentially as described before (Stiehl *et al.* 2006). Protein concentrations were determined by the Bradford method. Alternatively, total cell lysates were prepared to analyse γH2AX levels. Therefore, cells were collected in ice-cold PBS, lysed with 1% SDS, 50 mM Tris-HCl pH 6.8 and 10% glycerol, followed by sonification and boiling for 5 minutes. Protein concentrations were determined using the detergent-insensitive BCA-assay (Pierce, Perbio Science, Lausanne, Switzerland). Protein (50 to 80 μg) was separated by SDS-PAGE, electro-transferred onto PVDF membranes and probed with antibodies derived against DNA-PKcs (Ab-4 cocktail, Labvision, Fremont, CA), pATM (10H11.E12, Cell Signaling, Danvers, MA), pCHK1(133D3, Cell Signaling), pCHK2 (Cell Signaling), γH2AX (Upstate), HIF-1α (mAb69 and NB100-479, Novus), Ku70 (N3H10, Abcam, Cambridge, UK), Ku80 (QED Bioscience, San Diego, CA) and β-actin (Sigma). Bound antibodies were detected by respective secondary antibodies (Pierce) and visualized with ECL substrate (Pierce).

2.3.3.7 mRNA quantification.

Total RNA was extracted as described previously (Stiehl *et al.* 2006). Following reverse transcription (RT) of 5 μg total RNA, mRNA levels were quantified with 1% of the diluted cDNA reaction mix by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma) in combination with a MX3000P light cycler (Stratagene, La Jolla, CA). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein S12 mRNA was

determined and data expressed as ratios relative to S12 levels. Melting point analyses of amplified PCR products were performed after each run to verify specific amplification.

2.3.3.8 Transient transfections and reporter gene assays.

MEF cells were transfected using polyethyleneimine as described previously (Stiehl *et al.* 2006). In brief, 2 μg reporter plasmid were co-transfected with 100 ng of pRL-CMV (Promega). After 24 hours the cells were equally distributed onto 12-well plates and exposed to 20% or 0.2% oxygen for another 20 hours. After cell lysis with passive lysis buffer (Promega), dual luciferase activity was determined according to the manufacturer's instructions (Promega). To generate the pGL3-mPRKDC luciferase reporter gene construct, the 5'-region of mouse *PRKDC* was amplified from genomic DNA derived from MEF cells, using specific *Nhe*I-flanked forward (5'-CATCGCTAGCGCGCGACAAAAGAAATCTG-3') and *Xho*I-flanked reverse (5'-TGCACTCGAGATCACGCCGGCACCGCTTC-3') PCR primers. The products were ligated into *Nhe*I and *Xho*I sites of pGL3basic (Promega, Madison, WI). Sequencing confirmed that they were identical to the respective database entry (GenBank accession NT_039624), regardless of whether the genomic DNA was isolated from MEF-*Hif1a*$^{+/+}$*r*T or MEF-*Hif1a*$^{-/-}$*r*T. The pH3SVL hypoxia-inducible reporter gene has been described previously (34).

2.3.4 Results

2.3.4.1 Etoposide induces cell cycle arrest in MEFs irrespective of the presence of HIF-1α.

We had shown previously that chemosensitivity and radiosensitivity are increased in HIF-1α-deficient MEFs (Unruh *et al.* 2003). To investigate the underlying mechanism(s), colony formation, cell cycle distribution and p53 function were analyzed. As shown in Fig. 1A, treatment with increasing doses of etoposide reduced colony formation in HIF-1α-deficient MEFs, confirming our previous IC_{50} determinations using MTT assays (Unruh *et al.* 2003).

Because corrupted cell cycle control could explain the enhanced susceptibility to etoposide in HIF-1α-deficient MEFs, we next assessed their

ability to initiate cell cycle arrest in response to DNA damage. Therefore, the cells were treated with 1 μM etoposide for 1 hour and subsequently cultured for up to 12 hours. As shown in Fig. 1B, 6 hours after exposure to etoposide the percentage of cells with a duplicated genome increased from 31.7% to 44.5% in MEF-*Hif1a*$^{+/+}$*r*T and from 31.7% to 41.7% in MEF-*Hif1a*$^{-/-}$*r*T, suggesting accumulation at the G2/M-checkpoint. Of interest, 12 hours after etoposide administration, the fraction of cells in G2/M was almost restored to the initial values in both cell lines. However, a substantial increase of cells in G1 phase at this time point (55.9% compared to 42.8% in untreated MEF-*Hif1a*$^{+/+}$*r*T and 53.1% to 44.1% in MEF-*Hif1a*$^{-/-}$*r*T, respectively) was denoted, providing evidence for re-entry from the G2/M checkpoint into the cell cycle (Fig. 1B). No further fluctuations were observed at later time points and neither the generation time nor the cell cycle distribution differed between the two untreated MEF cell lines (data not shown).

Since the MEF cell lines used in this work were originally immortalized by stable expression of SV40 large T antigen and oncogenically transformed by infection with a recombinant retrovirus expressing activated H-*ras* 61L (Ryan *et al.* 2000), differential expression of these two genes might have caused differential chemosensitivity. However, as shown in Fig. 1C, no difference in large T antigen levels could be observed between the two cell lines. No human H-*ras* mRNA was detectable by RT-qPCR, suggesting genomic silencing of the stably integrated cDNA.

Finally, we considered differential functional inactivation of p53 by the large T antigen which might confound the response to DNA damage. However, after 8 hours of etoposide treatment a robust induction of the p53 target gene p21^{CIP1} was detected in both MEF lines, irrespective of the presence of HIF-1α or the cell culture oxygenation (Fig. 1D). The p53 target genes Bax and Noxa were similarly regulated (data not shown). The mRNA levels of the two cell cycle regulatory proteins p16^{INK4a} and p27^{KIP1} showed no marked differences in the two cell lines, while induction of the known pro-apoptotic HIF target gene BNIP3 confirmed hypoxia and HIF-1-dependent target gene activation (Fig. 1D). Thus, these data show that there are no

major differences in p53 inactivation in the two cell lines due to cell immortalization.

2.3.4.2 Increased accumulation of DNA-DSBs in HIF-1α-deficient MEF cells following etoposide treatment.

While etoposide might affect DNA integrity by several mechanisms, inhibition of topoisomerase II, leading to stalled replication forks, is thought to result in DNA-DSB in transcriptionally active cells (Burden and Osheroff 1998). Therefore, we applied alkaline single cell electrophoresis to quantify the DNA damage in MEF cells in response to treatment with etoposide. As reflected by the extended migration of fragmented DNA in comet tails, both cell lines showed an increase in DNA damage which dose-dependently correlated with the etoposide concentrations (Fig. 2A). At least 150 comet tails per experiment were quantified and DNA migration was expressed as *tail moment* (Fig. 2B). Of note, DNA migration was higher in MEF-*Hif1a*$^{-/-}$*r*T than in MEF-*Hif1a*$^{+/+}$*r*T in etoposide-treated as well as in untreated cells, suggesting HIF-1α-dependent changes in DNA integrity.

To characterize the nature of this increased susceptibility to DNA damage, aliquots of cells from the same experiments were analyzed by immunoblotting for phosphorylated histone 2AX (γH2AX), an established and sensitive marker of DNA-DSB. In line with the comet data, MEF-*Hif1a*$^{-/-}$*r*T also showed increased γH2AX (Fig. 2C). We next determined nuclear foci formation of p53-binding protein 1 (53BP1) and γH2AX at sites of DNA-DSB. Indeed, in response to doses of etoposide as low as 0.25 μM, increased overlapping immunofluorescence of 53BP1 and γH2AX was seen in MEF-*Hif1a*$^{-/-}$*r*T compared with MEF-*Hif1a*$^{+/+}$*r*T (Fig. 2D). These data suggest increased numbers of basal and inducible DNA-DSBs in HIF-1α-deficient MEFs.

2.3.4.3 Analysis of DNA-DSB repair pathways in MEF-*Hif1a*$^{+/+}$*r*T and MEF-*Hif1a*$^{-/-}$*r*T.

Two conserved major DNA-DSB repair pathways have been implicated in the response to DNA-DSB damage: homologous recombination (HR) and non-homologous end joining (NHEJ). When DNA-DSBs accumulate, ATM/ATR

and DNA-PKcs kinases are activated by phosphorylation. Each of these kinases preferentially phosphorylates specific targets, leading to cell cycle arrest by phosphorylation of checkpoint kinases (CHK1 and CHK2) and/or sustained recruitment of DNA repair proteins to the DNA-DSB foci via phosphorylation of H2AX. Upon treatment with etoposide, ATM Ser1981 autophosphorylation was strongly enhanced in both cell lines with a slightly higher degree in HIF-1α-deficient MEFs (Fig. 3A). However, while basal CHK1 Ser345 phosphorylation was slightly increased in MEF-*Hif1a*$^{-/-}$*r*T, Thr387 autophosphorylation of CHK2, a downstream event of ATM activation, showed similar signals in the two cell lines (Fig. 3A).

To identify the factor(s) involved in dysregulated DNA-DSB repair in HIF-1α-deficient MEF cells, candidate genes were screened by RT-qPCR of RNA derived from MEFs kept under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions. Transcript levels of PHD3 and CAIX were determined to control for hypoxia- and HIF-dependent gene expression (Stiehl *et al.* 2006), whereas growth arrest and DNA damage (GADD)153 mRNA was used as a HIF-independent hypoxia-inducible gene (Carrière *et al.* 2004). As shown in Fig. 3B, mRNA levels of 17 genes involved in DNA repair were measured. While none of these genes were regulated in a hypoxia-inducible manner, an up to 3-fold reduction in basal mRNA levels of members of the DNA-PK complex was detected in MEF-*Hif1a*$^{-/-}$*r*T cells (not visible in the scale chosen for Fig. 3B).

2.3.4.4 Dysregulation of DNA-PK complex members in HIF-1α-deficient MEF cells.

Because the previously reported HIF-dependent chemoresistance was oxygenation-independent (Unruh *et al.* 2003), we further evaluated the HIF-1α-dependent differences in basal DNA-PK expression. As shown in Fig. 4A, DNA-PKcs and Ku80 mRNA levels were significantly reduced in MEF-*Hif1a*$^{-/-}$*r*T when compared with MEF-*Hif1a*$^{+/+}$*r*T cells, independent of the oxygen concentration. In contrast, Ku70, ATM and ATR mRNA levels were not affected by the absence of HIF-1α. HIF-dependent and/or oxygen-dependent gene regulation was controlled in the same samples by monitoring BNIP3 and GADD153 mRNA levels, respectively. As shown in Fig. 4B, also the protein

levels of all three DNA-PK subunits, including Ku70, were reduced in MEF-*Hif1a*$^{-/-}$*r*T cells.

The *Mcm4* gene (minichromosome maintenance-deficient 4 homolog, also known as cell division control protein 21, *Cdc21*) is structurally organized in a head-to-head arrangement with the *DNA-PKcs* gene, only separated by a conserved CpG-rich promoter region of approx. 700 bp and 500 bp in humans and mice, respectively (Connelly *et al.* 1998; Saito *et al.* 1998). Interestingly, MCM4 mRNA is regulated in the same HIF-1α-dependent but oxygen-independent manner as DNA-PKcs (Fig. 4A), suggesting transcriptional co-regulation. To determine promoter activity, the shared 5' region of the mouse *PRKDC* and *MCM4* genes (a 695 bp DNA fragment spanning 16 bp 5' of the *MCM4* and 2 bp 3' of the *PRKDC* translational start sites) was amplified by PCR using genomic DNA derived from MEF-*Hif1a*$^{+/+}$*r*T and MEF-*Hif1a*$^{-/-}$*r*T cells. Of note, no sequence variations between the two cell lines were observed in this region. Transient transfections of a luciferase reporter gene driven by this promoter region revealed a substantial decrease in reporter gene activity in MEF-*Hif1a*$^{-/-}$*r*T when compared with MEF-*Hif1a*$^{+/+}$*r*T cells (Fig. 4C). Exposure of the transfected cells to 0.2% oxygen for 20 hours reduced reporter gene activity by about 50%, irrespective of the presence of HIF-1α. Parallel transfections of the two cell lines with the HRE-driven pH3SVL confirmed HIF-dependent hypoxic gene induction (Fig. 4C). Forced expression of exogenous HIF-1α in MEF-*Hif1a*$^{-/-}$*r*T failed to increase the activity of the co-transfected 695 bp mPRKDC promoter region, although transfected HIF-1α protein was clearly detectable by immunoblotting of parallel cultures (data not shown). While reduced promoter activity is sufficient to explain the decreased DNA-PKcs basal mRNA levels in HIF-1α-deficient MEFs, these data suggest that increased HIF-1α levels do not further induce mouse *PRKDC* gene expression, as expected from the lack of hypoxic induction of DNA-PKcs mRNA in wild-type MEF cells.

2.3.4.5 Cell model-specific effects of HIF-dependent chemoresistance.

To test our findings in an independent HIF-1α-deficient model, we generated HIF-1α mRNA knock-down cell lines by stable shRNA transfection in MEF-

Hif1a$^{+/+}$*r*T cells. However, partial HIF-1α mRNA knock-down only marginally affected the expression of DNA-PK complex members (data not shown). Therefore, we repeated our experiments in a HIF-1α-deficient MEF cell line derived from an independent knock-out mouse strain (Stiehl *et al.* 2006). These cell lines were named MEF-*Hif1a*$^{+/+}$T and MEF-*Hif1a*$^{-/-}$T since they were not transformed by H-*ras*. However, whereas MEF-*Hif1a*$^{-/-}$*r*T showed the expected increased susceptibility to etoposide, MEF-*Hif1a*$^{-/-}$T cells were equally sensitive to etoposide as the corresponding wild-type control (Fig. 5A).

In line with this finding, no difference in protein levels of DNA-PKcs could be detected between MEF-*Hif1a*$^{+/+}$T and MEF-*Hif1a*$^{-/-}$T, while DNA-PKcs was clearly lower in MEF-*Hif1a*$^{-/-}$*r*T than in MEF-*Hif1a*$^{+/+}$*r*T (Fig. 5B, left panel). To control for the genotype of the MEF-*Hif1a*$^{-/-}$T cells, HIF-1α immunoblotting of nuclear extracts was included. Interestingly, following prolonged exposure there was an immunoreactive truncated but still hypoxia-inducible form of HIF-1α protein detectable in MEF-*Hif1a*$^{-/-}$T but not in MEF-*Hif1a*$^{-/-}$*r*T (Fig. 5B, right panel). This truncated HIF-1α protein cannot be directly transcriptionally active since it is derived from a targeted deletion of exon 2, containing the bHLH DNA-binding region of HIF-1α. Indeed, protein levels of the HIF target gene PHD2 were no longer hypoxically induced in both HIF-1α-deficient cell lines. Finally, the levels of SV40 large T antigen were similar in all 4 cell lines, ruling out major differences due to changes in large T levels. Another major difference between the two cell lines might be the H-*ras* levels. However, using RT-qPCR we could not detect any H-*ras* mRNA, neither in the MEF-*Hif1a*$^{+/+}$T/MEF-*Hif1a*$^{-/-}$T nor in the MEF-*Hif1a*$^{+/+}$*r*T/MEF-*Hif1a*$^{-/-}$*r*T cell lines, while diluted H-*ras* plasmid cDNA was still detectable (data not shown).

To further confirm lack of HIF-dependent transcriptional activity, MEF-*Hif1a*$^{+/+}$T and MEF-*Hif1a*$^{-/-}$T cells were transiently transfected with the HRE-driven pH3SVL reporter plasmid. As expected, there was no residual hypoxic induction of HIF-dependent reporter gene expression in MEF-*Hif1a*$^{-/-}$T (Fig. 4C). Similar results were obtained for the endogenous transcripts of BNIP3 which were only induced in HIF-1α-containing MEF-*Hif1a*$^{+/+}$T (Fig. 4D). Induction of GADD153 in both cell lines confirmed their hypoxic status.

2.3.5 Discussion

Due to microenvironmental changes as well as oncogenic transformation, HIF-1α levels are constitutively increased in solid tumors. In this work we established a new molecular link between HIF-1α and tumor therapy resistance by demonstrating that both basal and etoposide-induced DNA damage is increased in HIF-1α-deficient cells. The DNA-PK complex members DNA-PKcs and Ku80 were found to be downregulated in HIF-1α-deficient cells on the mRNA levels. On the protein level, not only DNA-PKcs and Ku80 but also the third DNA-PK member Ku70 was decreased in HIF-deficient cells. The reduced Ku70 protein levels, despite similar mRNA levels, are likely explained by the lack of mutual protein stabilization of the three DNA-PK complex members: radiosensitive CHO cells expressing reduced levels of Ku80 also showed lowered levels of Ku70 and forced expression of Ku86 in such cells concomitantly restored Ku70 protein levels (Errami *et al.* 1996; Singleton *et al.* 1997). Importantly, defective DNA-PK is known to lead to increased efficiency in tumor therapy, including to etoposide treatment (Lees-Miller *et al.* 1995; Zhao *et al.* 2006).

Supporting our results using HIF-1β-deficient hepatoma cells, Um and colleagues reported induction of DNA-PKcs, Ku70 and Ku80 by hypoxia and iron chelation in a HIF-1β-dependent manner (Um *et al.* 2004). While Ku has been reported to be induced by hypoxia in other cell types as well (Ginis and Faller 2000; Lynch *et al.* 2001), we could not observe any hypoxia-inducible Ku or DNA-PKcs expression in MEF cells. How could a HIF-dependent but non-hypoxia-inducible gene expression be explained? Genetically HIF-1α-deficient cells are well known to have even lower HIF target gene levels than wild-type cells when both cell lines are cultured under the standard "oxic" 20% oxygen conditions (Iyer *et al.* 1998). This finding is remarkable since HIF-1α protein is usually below the detection limit of standard methods in subconfluent cells cultured at 20% oxygen, which in principle should suffice to completely degrade HIF-1α. A new hint to the mechanism of "oxic" HIF-1α function was provided by the interesting finding that a HIF-1α knock-out-derived MEF cell line that still contained a truncated version of HIF-1α neither showed increased chemosensitivity nor increased DNA-PKcs protein levels.

Thus, only the complete absence of HIF-1α in MEF cells conferred "oxic" chemosensitivity and residual HIF-1α might be sufficient to reverse this effect. In line with this conclusion, partial downregulation of HIF-1α by shRNA interference only weakly affected chemoresistance in MEF-*Hif1a*$^{+/+}$*r*T cells, while hypoxic induction of HIF-1α target genes was strongly impaired (data not shown). DNA-binding activity appears not to be required, ruling out a direct transcriptional function. Indeed, despite the presence of putative HRE sites, the DNA-PKcs promoter was not found to be induced by hypoxia. Rather, the DNA-PKcs promoter as well as endogenous DNA-PKcs mRNA expression were downregulated by the (complete) absence of HIF-1α.

The concept of "oxic" HIF-1α was further developed by Giaccia and co-workers who showed that loss of HIF-1α under aerobic conditions accelerated cellular senescence in a macrophage migration inhibitory factor (MIF)-dependent manner (Welford *et al.* 2006). The *VE-cadherin* gene has been reported to be induced by HIF-2 but not by hypoxia (Le Bras *et al.* 2007). Finally, Zeng and colleagues recently reported that HIF-1α decreased the sensitivity of lung cancer cells to paclitaxel under normoxic conditions, further supporting our data (Zeng *et al.* 2007). Thus, these examples demonstrate that the low concentrations of HIF-1α under normoxic conditions are sufficient for certain cellular processes. Translated to tumor biology, this could imply that slight changes in HIF-1α mRNA expression levels by oncogenic transformation early in tumor formation might already lead to changes in HIF target gene expression, even before the growing tumor creates severely hypoxic regions with massively induced HIF-1α protein.

In apparent contrast to our findings, Huang and co-workers reported that HIF is responsible for genomic instability by downregulating several factors involved in DNA repair (Huang *et al.* 2007). However, only treatments that cause DNA-DSBs were effectively impaired by HIF in our model system (20). While DNA-DSB repair by NHEJ confers resistance to DNA-DSB-causing agents, NHEJ is an error-prone process. Thus, HIF-dependent DNA-DSB repair might even boost genomic instability and malignant progression.

2.3.6 Acknowledgements

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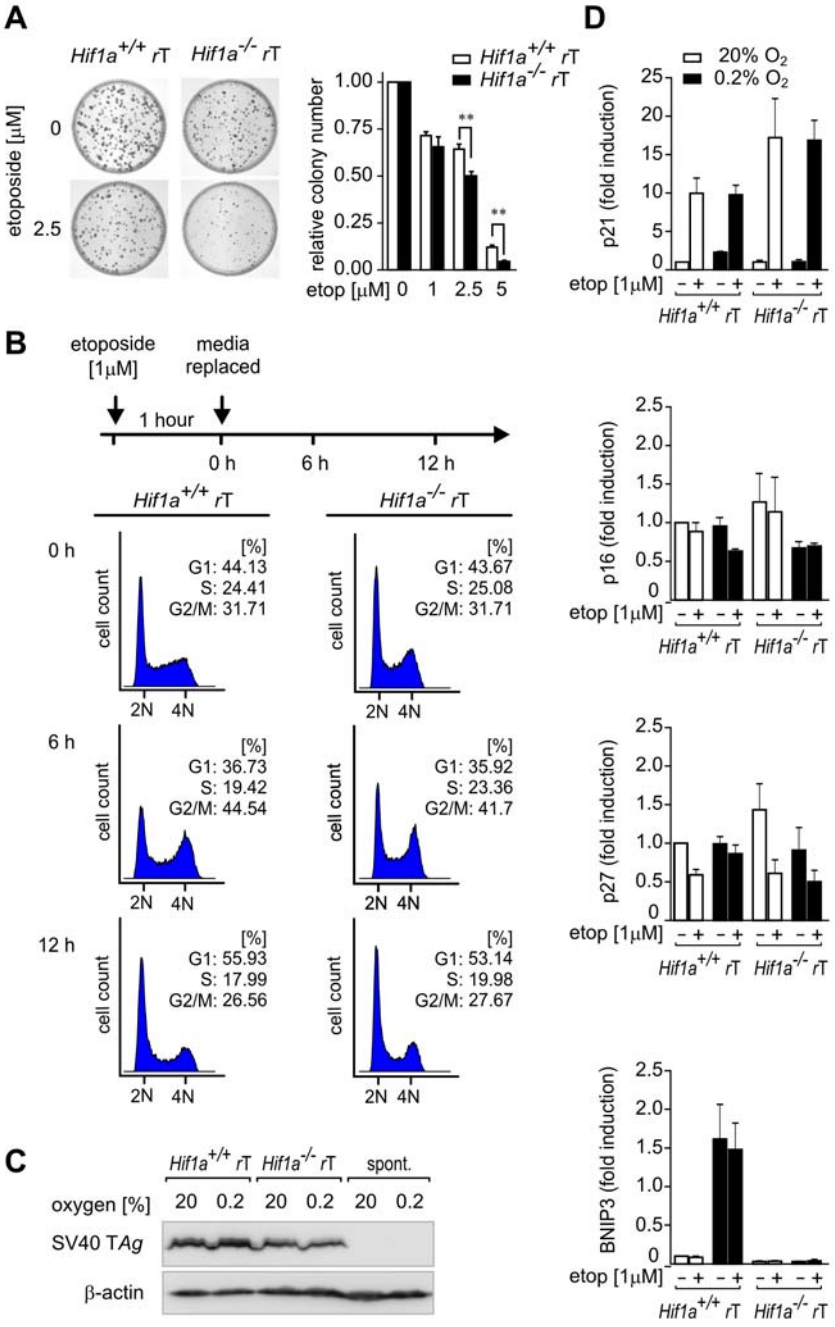

Figure 1. Etoposide-induced cell cycle arrest in wild-type and HIF-1α-deficient MEFs. *A*, MEF-*Hif1a*$^{+/+}$*r*T and MEF-*Hif1a*$^{-/-}$*r*T were treated with 1, 2.5 and 5 μM etoposide for 1 hour, trypsinized and plated in triplicates for each concentration. Colonies were counted after 6 days and normalized to the untreated control plates. Shown are mean values + SEM of a representative experiment performed in triplicates. Asterisks indicate statistically significant differences (** $p < 0.01$) as calculated by student's t-test. *B*, Cell cycle distribution of MEF-*Hif1a*$^{+/+}$*r*T and MEF-*Hif1a*$^{-/-}$*r*T was determined by FACS analysis following treatment with 1 μM etoposide as indicated. *C*, MEF lysates were immunoblotted for SV40 large T antigen and β-actin. Spontaneously immortalized MEFs (spont.) served as negative controls. *D*, MEFs were treated with 1 μM etoposide for 8 hours at either 20% or 0.2% oxygen and p21, p16, p27 and BNIP3 mRNA levels were quantified by RT-qPCR. Values were normalized to the mRNA levels of the housekeeping ribosomal protein s12. Mean values + SEM of 3 independent experiments are shown.

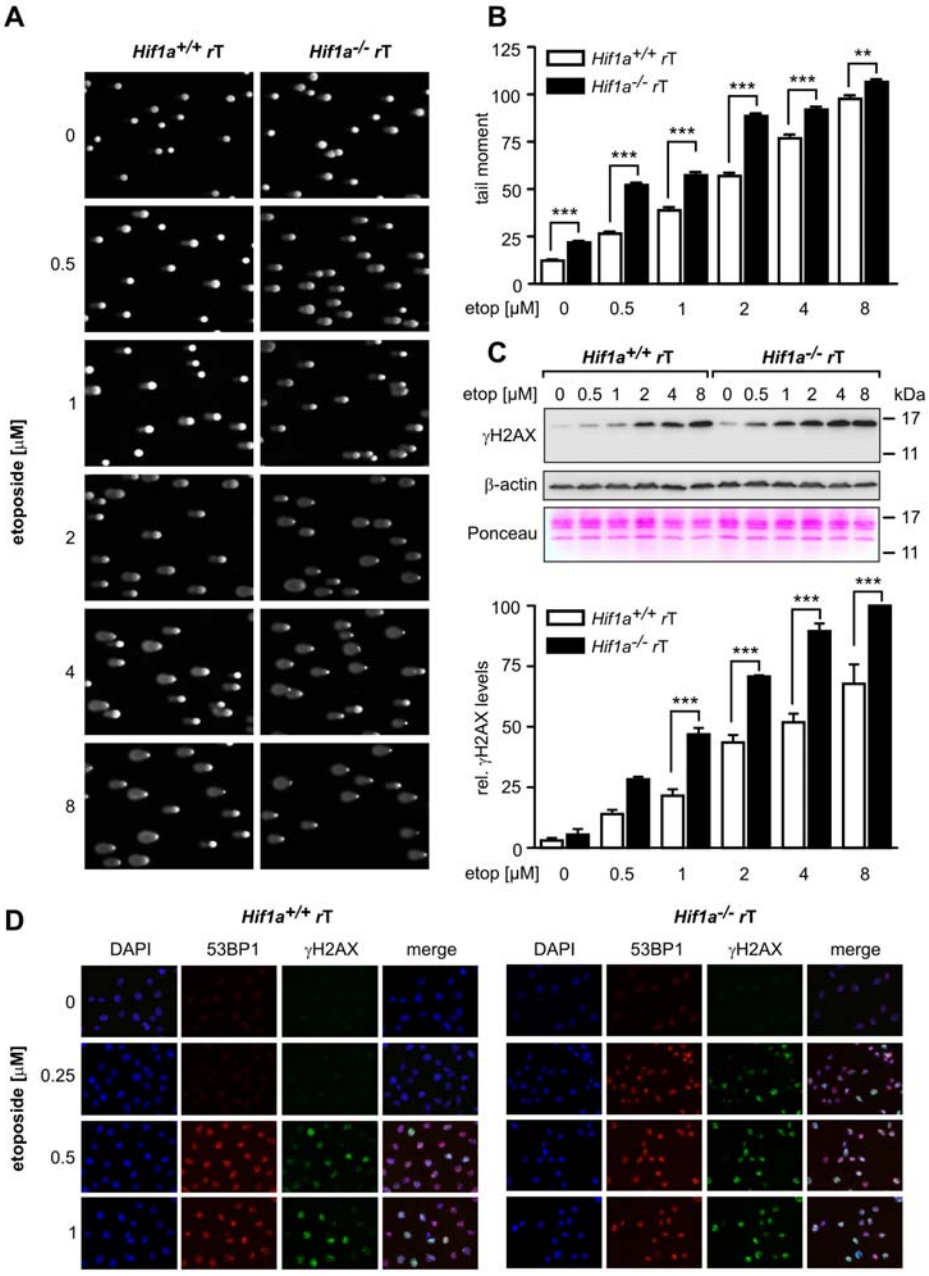

Figure 2. DNA fragmentation in wild-type and HIF-1α-deficient MEFs after a genotoxic insult. *A*, DNA fragmentation in MEF-*Hif1a*$^{+/+}$*r*T and MEF-*Hif1a*$^{-/-}$*r*T cells was quantified by single-cell electrophoresis following exposure to increasing amounts of etoposide for 1 hour. DNA was stained with SYBR green and all images were acquired with fixed exposure times. *B*, DNA fragmentation was quantified by determining the tail moment of at least 150 comets per condition using CometScore software. *C*, Aliquots of cell cultures from *A* were subjected to immunoblotting for γH2AX. Band intensity was quantified by densitometry and normalized to β-actin levels. Ponceau staining served to demonstrate equal extraction of histones. *D*, MEF cells were grown on Iumox membranes and treated with up to 1 µM etoposide for 1 hour. Immunofluorescence was performed using antibodies directed against 53BP1 and γH2AX and nuclei were counterstained with DAPI. All data are given as mean values + SEM and asterisks indicate statistically significant differences (** p<0.01, *** p<0.001) as calculated by Tukey's multiple comparison test (*B*, *C*).

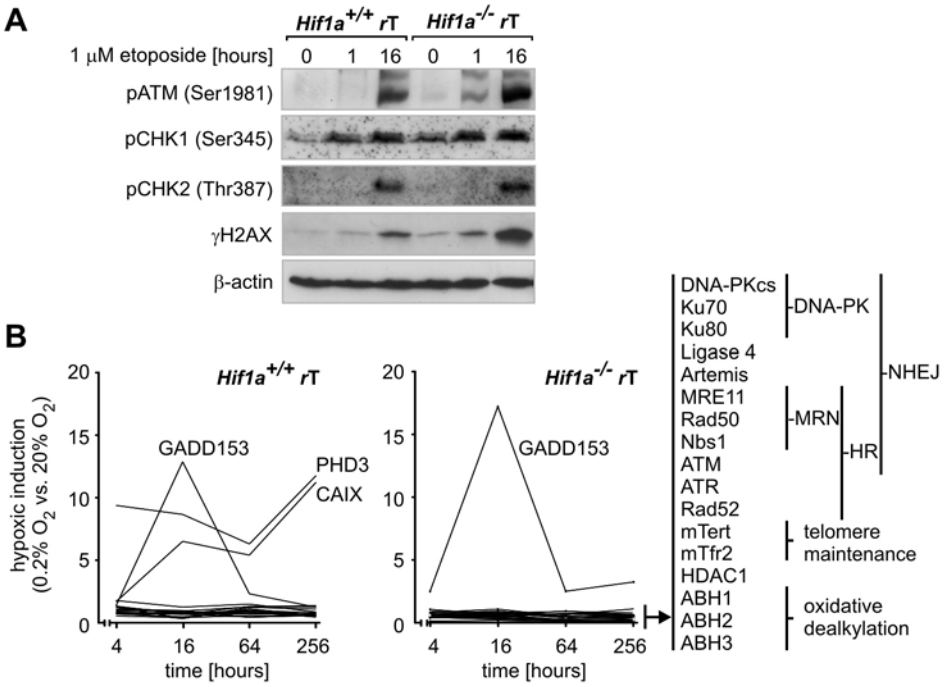

Figure 3. Analysis of DNA repair pathways in wild-type and HIF-1α-deficient MEFs. *A*, MEF-*Hif1a*$^{+/+}$*r*T and MEF-*Hif1a*$^{-/-}$*r*T cells were treated with 1 μM etoposide for 0 to 16 hours under normoxic conditions. Phosphorylation of ATM, CHK1, CHK2 and H2AX was analyzed by immunoblotting using phosphoprotein-specific antibodies. β-actin served as loading control.

B, MEF-*Hif1a*$^{+/+}$*r*T and MEF-*Hif1a*$^{-/-}$*r*T cells were permanently cultured at 20% or 0.2% O_2 for 0, 4, 16, 64 or 256 hours in a hypoxic work station. Transcript levels of 17 candidate genes involved in DNA repair were quantified by RT-qPCR. Hypoxic induction factors are shown following normalization to the mRNA levels of ribosomal protein S12.

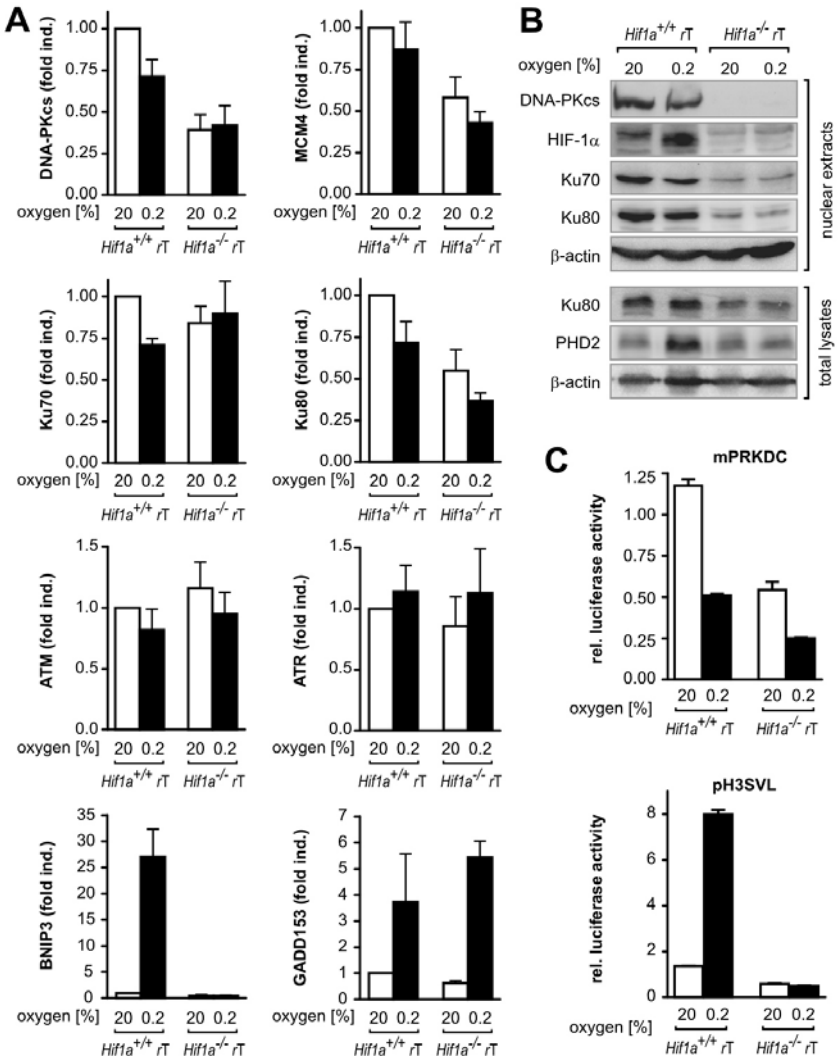

Figure 4. Reduced expression of DNA-PK subunits in HIF-1α-deficient MEFs. *A*, mRNA levels of the three DNA-PK subunits DNA-PKcs, Ku70 and Ku80, as well as MCM4, ATM and ATR, were quantified in MEF-*Hif1a*$^{+/+}$*r*T and MEF-*Hif1a*$^{-/-}$*r*T cells after exposure for 16 hours to 0.2% oxygen. BNIP3 and GADD153 mRNA levels were determined as controls. All values were normalized to ribosomal protein s12 mRNA levels. Mean induction factors +SEM of 3 independent experiments performed in triplicates are shown. *B*, Immunoblot analyses of DNA-PKcs, Ku70 and Ku80 in nuclear extracts of MEF cells treated as above. HIF-1α protein levels were determined to control for hypoxic induction and genotype. β-actin levels were determined as loading control. Reduced levels for Ku80 were confirmed in total cell lysates (bottom panel) and HIF-dependent hypoxic induction of PHD2 is shown. *C*, Relative luciferase activities were determined in MEF cells transiently co-transfected with 2 μg pGL3-mPRKDC or pH3SVL and 0.1 μg pRL-CMV as transfection control. Transfected cultures were incubated for 20 hours at either 20% or 0.2% oxygen and dual luciferase activity was measured. Data are shown as mean values of hexaplicates +SEM.

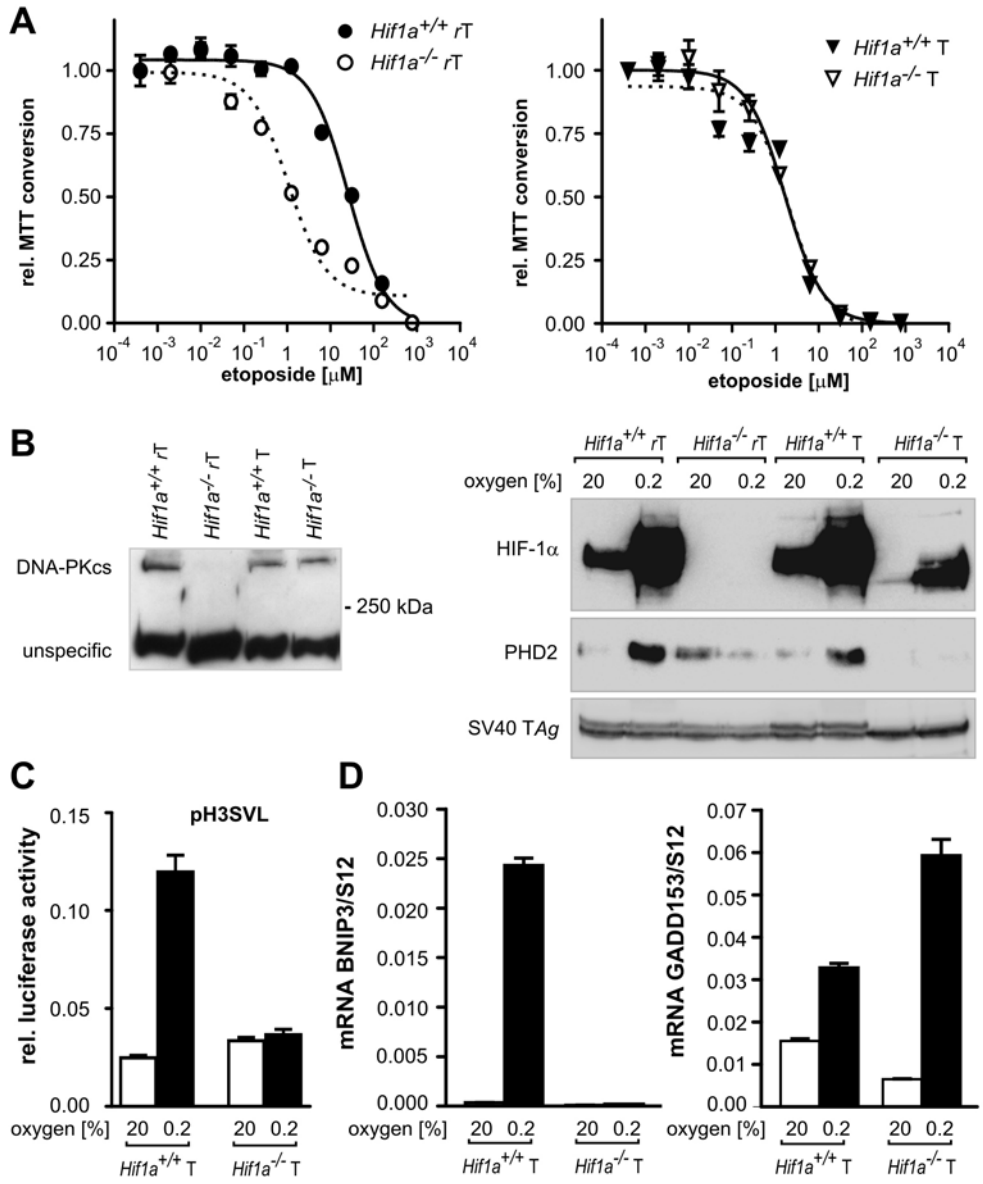

Figure 5. Increased chemosensitivity to etoposide is not observed in HIF-1α knock-out MEFs containing residual truncated HIF-1α. A, Relative MTT conversion in response to etoposide treatment for 24 hours was determined in two independent MEF cell lines distinguished by the presence (rT) or absence (T) of H-ras transfection. Cultures were treated in triplicates and values normalized to untreated control cells. Data are given as mean values of triplicates + SEM. B, Nuclear protein was subjected to immunoblot analysis of DNA-PKcs (left) or HIF-1α, PHD2 and SV40 large T antigen protein levels. C, MEF-*Hif1a*$^{+/+}$T and MEF-*Hif1a*$^{-/-}$T were transiently co-transfected with 2 μg pH3SVL and 0.1 μg pRL-CMV as transfection control. Dual luciferase activity was measured and values expressed as mean of hexaplicates + SEM. D, BNIP3 and GADD153 mRNA levels were quantified by RT-qPCR in MEF-*Hif1a*$^{+/+}$T and MEF-*Hif1a*$^{-/-}$T after exposure for 16 hours to 0.2% oxygen. All values were normalized to ribosomal protein s12 mRNA levels and are shown as mean + SEM of experiments performed in triplicates.

2.4 Induction of the HIF system by low levels of HSP90 inhibitors[1]

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2.4.1 Abstract

The heterodimeric hypoxia-inducible factor (HIF)-1 is involved in key steps of tumor progression and therapy resistance and thus represents an attractive anti-tumor target. Since HSP90 plays an important role in HIF-1α protein stabilization and because HSP90 inhibitors are currently being tested in clinical phase I trials for anti-cancer treatment, we investigated their role as anti-HIF-1α agents. Surprisingly, low dose (5–30 nM) treatment of HeLa cells with three different HSP90 inhibitors (17-AAG, 17-DMAG and GA) increased HIF-1-dependent reporter gene activity, whereas higher doses (1-3 μM) resulted in a reduction of hypoxia-induced HIF-1 activity. In line with these data, low-dose treatment with HSP90 inhibitors increased and high-dose treatment reduced hypoxic HIF-1α protein levels, respectively. HIF-1α protein stabilized by HSP90 inhibitors localized to the nucleus. As a result of HSP90-modulated HIF-1 activity, the levels of the tumor relevant HIF-1 downstream targets CAIX, PHD3 and VEGF were increased or decreased after low-dose or high-dose treatment, respectively. Bimodal effects of 17-AAG on vessel formation were also seen in the chick chorioallantoic membrane angiogenesis assay. In summary, these results suggest that dosage will be a critical factor in the treatment of tumor patients with HSP90 inhibitors.

2.4.2 Introduction

The hypoxia-inducible factor-1 (HIF-1) activates a number of oxygen-regulated genes critically involved in adaptation to hypoxia (Semenza 2000; Wenger 2002; Kaelin 2005). Under normoxic conditions, the von Hippel-Lindau tumor suppressor protein (pVHL) targets the HIF-1α subunit for rapid ubiquitination and proteasomal degradation (Maxwell *et al.* 1999). Binding of the pVHL tumor suppressor protein requires modification of HIF-1α by a family of low-affinity oxygen-dependent prolyl-4-hydroxylase domain proteins (PHD) (Epstein *et al.* 2001; Ivan *et al.* 2001; Jaakkola *et al.* 2001). Three HIF-1α modulating PHDs, i.e. PHD1, 2, and 3, have been described so far.

HIF-1 is constitutively upregulated in many cancer types and plays a major role in tumor progression (Semenza 2002; Brown and Wilson 2004; Vaupel 2004); HIF-1 elevates vascular endothelial growth factor (VEGF)-dependent tumor angiogenesis, it regulates tumor acidosis by increasing carbonic anhydrase IX (Wykoff *et al.* 2000), and it mediates the increased glycolytic capacity of tumor cells, known as Warburg effect (Seagroves *et al.* 2001). Moreover, we and others have recently shown that HIF-1 confers resistance against chemo- and radiotherapy (Unruh *et al.* 2003; Williams *et al.* 2005). Since HIF-1 regulates key steps of tumor progression and therapy resistance it represents an attractive anti-tumor target. Anti-HIF-1 effects have been described for a variety of agents, including several drugs already in clinical use such as taxol, topotecan or the HSP0-inhibiting ansamycin derivatives (for review see (Giaccia *et al.* 2003; Yeo *et al.* 2004)). The mechanism by which taxol and topotecan destabilize HIF-1α is currently unknown. With regard to ansamycin derivatives, we and others have previously shown that HSP90 is important for hypoxic stabilization of HIF-1α (Isaacs *et al.* 2002; Katschinski *et al.* 2002; Zhou *et al.* 2004). In HSP90β-deficient cells, hypoxic stabilization of HIF-1α was significantly delayed compared to wild type cells (Katschinski *et al.* 2004). A similar effect could be shown by inhibiting HSP90 with the ansamycin derivative geldanamycin (GA), suggesting that HSP90 is required for the rapid hypoxic stabilization of HIF-1α which otherwise might be degraded by unspecific pathways (Katschinski *et al.* 2004). To inhibit the HIF system, doses in the micromolar range have usually been used (Katschinski

et al. 2002; Mabjeesh *et al.* 2002; Isaacs *et al.* 2004). Besides HIF-1α a variety of other classical HSP90 client proteins have been demonstrated to be targeted by ansamycin derivatives, including v-src, FAK, ErbB2 and Akt kinase (Goetz *et al.* 2003). Since the clinical application of GA is restricted due to severe side effects, modified ansamycin derivatives like 17-AAG or the water soluble 17-DMAG have been developed (Sausville 2004). The safety of 17-AAG application in humans was demonstrated in three recently published clinical phase I trials (Banerji *et al.* 2005; Goetz *et al.* 2005; Ramanathan *et al.* 2005).

Because it is difficult to control the local dose of an intravenously or orally applied drug within the tumor tissue, we have determined the effect of GA, 17-AAG and 17-DMAG on the HIF-system over a wide dose range. Surprisingly, an increase in HIF-1α protein levels as well as HIF-target gene expression was found for all three compounds following application in the low nanomolar range, whereas higher doses efficiently downregulated the HIF-system. These results should be considered when HSP90 inhibitors are to be tested for anti-tumor therapy.

2.4.3 Materials and Methods

2.4.3.1 Chemicals

Geldanamycin (GA), 17-demethoxy-17((2-(dimethylamino)ethyl)amino) geldanamycin hydro-chloride (17-DMAG) and 17-allylamino-17-demethoxygeldanamycin were a kind gift of Kosan Biosciences Inc. (Hayward, CA). All three drugs were dissolved in DMSO to generate 2 mM stock solutions. All further dilutions were prepared in cell culture medium. All other chemicals were obtained from Sigma (Taufkirchen, Germany) or Roth (Karlsruhe, Germany).

2.4.3.2 Cell lines and cell culture

All cell lines were cultured in Dulbecco's modified Eagle's medium (high glucose) as described previously. Oxygen partial pressures in the hypoxic incubator (Binder, Tuttlingen, Germany) were either 140 mm Hg (20% O_2 vol/vol, normoxia) or 7 mm Hg (1% O_2 vol/vol, hypoxia).

2.4.3.3 Transient transfections

HeLa and Hep3B cells were transiently transfected with the HIF-dependent firefly luciferase reporter gene construct pH3SVL, containing a total of 6 HIF-1 DNA-binding sites derived from the transferrin gene by the calcium phosphate co-precipitation method as described previously (Rolfs *et al.* 1997). HeLa or Hep3B cells were seeded in 24 well plates at a concentration of 5 x 10^4 cells/well. One day after seeding, cells were co-transfected with 0.25 µg pH3SVL together with 0.04 µg renilla luciferase control plasmid pRL-SV40 (Promega, Mannheim, Germany). Cells were treated with HSP90 inhibitors dissolved in DMSO or DMSO alone and subsequently exposed to normoxic or hypoxic conditions for 24 hours. Luciferase activities were determined using the dual-luciferase assay kit (Promega, Mannheim, Germany). Results were normalized to the solvent-treated normoxic control values which were arbitrarily defined as 1.

2.4.3.4 Protein extraction and immunoblot analyses

Combined cytoplasmic and nuclear extracts of cultured cells were prepared using 0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.1% NP-40. Protein concentrations were determined by the Bradford method using BSA as a standard. For immunoblot analysis, 50 µg cellular protein was electrophoresed through 7.5% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Amersham, Freiburg, Germany) by semi-dry blotting (BioRad, München, Germany). Membranes were stained with Ponceau S (Sigma) to confirm equal protein loading and transfer. HIF-1α, HSP70, HSP90, Akt, HSF-1 and β-actin were detected using the following antibodies: mouse monoclonal anti-HIF-1α IgG_1 (Transduction Laboratories, Heidelberg, Germany), mouse monoclonal anti-HSP70 IgG_1 (StressGen, Victoria, Canada), rat monoclonal anti-HSP90 IgG_{2a} rabbit (StressGen, Victoria, Cancada), rabbit polyclonal anti-Akt (Cell Signaling Tehnology, Beverly, USA), polyclonal anti-HSF-1 (Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin IgG_1 (Sigma) followed by the appropriate secondary horseradish peroxidase-conjugated polyclonal antibodies raised in goats (Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescence

detection of horseradish peroxidase was performed by incubation of the membranes with 100 mM Tris-HCl (pH 8.5), 2.65 mM H_2O_2, 0.45 mM luminol and 0.625 mM coumaric acid (all purchased from Sigma) for 1 minute followed by exposure to X-ray films (Amersham).

2.4.3.5 HIF-1α immunofluorescence

Cells were fixed with methanol for 5 minutes. The non-specific binding sites were blocked with 3% BSA in PBS for 30 minutes. The cells were incubated for 1 hour with a mouse monoclonal anti-HIF-1α IgG_1 antibody (Transduction Laboratories) diluted 1:10 in 3% BSA in PBS followed by a TRITC-coupled secondary anti-mouse (Dako) antibody diluted 1:200 with 3% BSA in PBS. Subsequently, all cells were stained with Hoechst 33258 dye for 5 minutes. After extensive washings with PBS, the slides were mounted and analyzed by fluorescence microscopy (Axioplan 2000, Carl Zeiss Vision, Mannheim, Germany).

2.4.3.6 RNA extraction and real-time RT-PCR

Hep3B cultures were grown on 6 well plates for 24 hours and total RNA was extracted. First strand cDNA synthesis was performed with 1 μg of RNA using the first strand synthesis kit from Fermentas (Fermentas, St. Leon-Rot, Germany). Subsequently, mRNA expression levels for CAIX, PHD1, PHD2, and PHD3 were quantified with 1 μl of cDNA reaction by real-time RT-PCR using a SybrGreen Q-PCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To verify RNA integrity and equal input levels, ribosomal protein L28 mRNA was determined and data expressed as ratios relative to L28 levels. Primers were as follows: hCAIX forward, 5'-gggtgtcatctggactgtgtt-3'; hCAIX reverse, 5'-cttctgtgctgccttctcatc-3'; PHD2 forward, 5'-gaaagccatggttgcttgtt-3'; PHD2 reverse, 5'-ttgccttctggaaaaattcg-3'; PHD3 forward, 5'-atcgacaggctggtcctcta-3'; PHD3 reverse, 5'-cttggcatcccaattcttgt-3'; hL28 forward, 5'-gcatctgcaatggatggt-3'; hL28 reverse, 5'-tgttcttgcggatcatgtgt-3'.

2.4.3.7 Chick embryo chorioallantoic membrane (CAM) assay

CAM assays were performed as described before (Linden *et al.* 2003). In brief, fertilized eggs (purchased from Lohmann Tierzucht, Cuxhaven, Germany) were incubated at 37°C in a humidified atmosphere. The eggs were kept on their sides and turned upside down twice a day. After 3 days, a small hole was drilled through the shell into the air sac (visualized using a strong light source), 2 ml of egg white were aspirated and a window of approx. 0.5 cm^2 was sawed into the side of the egg. The window was sealed with tape, and the eggs were reincubated until day 10, when 17-AAG was applied to the CAM using the Elvax method. Therefore, ethylene/vinyl acetate copolymer beads (elvax 40L-03, DuPont,Wilmington, DE; kindly provided by C. H. Erbslöh KG, Krefeld, Germany) were extensively washed in ethanol and dried under vacuum. The Elvax beads were then dissolved in methylene chloride at 10% (w/v), and 17-AAG was added to the desired concentration. One drop (40 µl) of this solution was pipetted onto a siliconized glass slide, and the solvent was allowed to evaporate completely. Using foreceps, the Elvax disk was carefully lifted from the glass slide and placed onto the CAM. At day 13, the window was enlarged and the CAM was documented using a digital camera (Olympus, Hamburg, Germany) coupled to an ocular of a stereomicroscope.

2.4.4 Results and Discussion

2.4.4.1 Modulation of HIF-1activity is highly dependent on HSP90 inhibitor concentration

HeLa cells were treated with 17-AAG, 17-DMAG or GA at concentrations ranging from 5 nM to 3 µM under normoxic or hypoxic conditions for 4 hours. We first demonstrated the efficiency of the HSP90 inhibitors in HeLa cells by determining the concentration of the heat shock transcription factor-1 (HSF-1). HSF-1 mediates the increased expression of HSPs following induction of a heat shock response with HSP90 inhibitors as reported previously (Sittler *et al.* 2001; McLean *et al.* 2004). In non-stressed HeLa cells grown at 37°C, HSF-1 was detected as an approx. 85 kDa protein by immunoblotting (Fig. 1). After treating HeLa cells for 4 hours at 42°C, however, the HSF-1 appeared

as an approx. 95 kDa protein. This shift in molecular mass most likely corresponds to the phosphorylation of HSF-1 and hence indicates the extent of its activation. A similar shift in molecular mass was observed after treating HeLa cells with increasing concentrations of 17-AAG (Fig. 1). Similar effects were seen after treatment with 17-DMAG and GA (data not shown), demonstrating that the HSP90 inhibitors were fully functional and confer a heat-shock-like response.

To explore whether the HSP90 inhibitors affect HIF-1 activity, transient transfections were performed with a HIF-1-dependent reporter gene. After simultaneous treatment with hypoxia and increasing concentrations (5 nM – 3 μM) of 17-AAG, 17-DMAG or GA, luciferase activity was measured as a marker for HIF-1 transcriptional activity (Fig. 2A to C). Hypoxia alone resulted in a roughly 50-fold induction of HIF-1 activity compared to normoxia. Surprisingly, treatment of HeLa cells with 5 nM or 30 nM of 17-AAG or GA resulted in a significant increase of hypoxia-mediated HIF activity. A similar effect was observed after treating the cells with 5 nM 17-DMAG. However, treatment with higher concentrations, i.e. 1-3 μM 17-AAG, 17-DMAG or GA, significantly decreased reporter gene activity. To rule out a cell line specific effect, Hep3B cells were treated with 17-AAG, 17-DMAG or GA (Fig. 2D). Like in HeLa cells, low-dose treatment with 5 nM of all three drugs resulted in an increased activity of HIF-1α, whereas the activity was decreased after treatment with 3 μM

2.4.4.2 Bimodal effect of HSP90 inhibitors on HIF-1α protein levels

Exposure of HeLa cells to 1% oxygen for 2-24 hours resulted in hypoxic stabilization of HIF-1α (Fig. 3). Hypoxic stabilization was strongest after 4 hours incubation at 1% O_2, whereas at later time points, i.e. 12 hours and 24 hours, the hypoxic stabilization was less pronounced. Treatment with 5 nM or 3 μM 17-AAG effected a heat shock-like response with increased expression of HSP70 and to a minor extent HSP90. This effect was more pronounced after treatment with 3 μM compared to 5 nM. In addition, the expression of HSPs increased with duration of 17-AAG treatment. In line with the decreased HIF-1 transcriptional activtiy and as described previously by us and others,

treatment with high doses of 17-AAG (3 μM) decreased hypoxic HIF-1α protein accumulation (Isaacs *et al.* 2002; Katschinski *et al.* 2002; Mabjeesh *et al.* 2002; Isaacs *et al.* 2004). However, consistent with the increased HIF-1 transcriptional activity, treatment with low doses of 17-AAG (5 nM) further increased the hypoxic stabilization of HIF-1α most prominentlyat early time points and to a lesser extent after 24 hours incubation. This may be explained by the fact that 17-AAG is accumulating intracellularly with time (Chiosis *et al.* 2003). The affinity of the isolated HSP90 protein for ansamycins has been determined to be in the low micromolar range (Panaretou *et al.* 1998; Roe *et al.* 1999). However, since ansamycins accumulate intracellularly, even nanomolar concentrations may be effective to reach this level inside the cells. In contrast to the treatment with 5 nM 17-AAG, the effect with 3 μM 17-AAG on HIF-1α accumulation as well as the full heat shock-like response were seen at later time points. Decreased levels after treatment with 3 μM but no increase with 5 nM 17-AAG were seen for the HSP90 client protein Akt, demonstrating that the dose-dependent modulation of HIF-1α is not a general phenomenon of all HSP90 client proteins. The PI3 kinase/Akt pathway is involved in HIF-1α stabilization (Zundel *et al.* 2000). Since Akt was downregulated after 3 μM 17-AAG treatment, this effect may be involved in the downregulation of HIF-1α after high dose 17-AAG treatment and may explain partly the time- and dose-dependent effects on the HIF-system. Other signal transduction pathways involved in the stabilization or activation of HIF-1α especially in cancer have also been described to be affected by HSP90 inhibitor treatment. In this respect it is interesting to note that erbB2 as well as the Raf/Ras/MAP kinase pathways, which are involved in HIF-1α stabilization and activation, have likewise been described to be affected by HSP90 inhibition(Goetz *et al.* 2003; Zhang and Burrows 2004; Peng *et al.* 2005)

2.4.4.3 HIF-1α protein induced by HSP90 inhibitors localizes to the nucleus

We next determined whether HSP90 inhibitors affect the nuclear translocation of hypoxia-induced HIF-1α protein. Therefore, HeLa cells were treated with 1% O_2 with or without addition of 5 nM or 3 μM 17-AAG for 4 hours. As shown

by indirect immunofluorescence analysis, HIF-1α protein accumulated exclusively within the nucleus under hypoxic conditions. Addition of 5 nM 17-AAG did not affect nuclear translocation of HIF-1α whereas 3 μM 17-AAG reduced HIF-1α protein levels (Fig. 4). Similar results were obtained by treating HeLa cells with GA or 17-DMAG (data not shown).

2.4.4.4 HSP90 inhibitors modulate HIF-dependent target gene expression involved in hypoxia adaptation

Modulation of HIF-1α protein expression and HIF-1 transcriptional activity by HSP90 inhibitors suggest that these drugs may affect pathways involved in tumor progression. Therefore, we investigated the effects of 17-AAG on the expression of the HIF-1 target gene CAIX (Grabmaier *et al.* 2004). CAIX has a major role in regulating proton flux and blockade of CAIX results in increased cell death under severe hypoxia, indicating an important mechanism of hypoxic adaptation (Potter and Harris 2004). Exposure of Hep3B cells for 24 hours at 1% O_2 indeed resulted in a strong increase in CAIX mRNA levels compared to cells grown at 20% O_2 (Fig. 5). Low-dose (5 nM) 17-AAG enhanced CAIX mRNA expression at 20% O_2 and even more pronounced at 1% O_2 ($p<0.05$), whereas treatment with 3 μM 17-AAG resulted in a significant decrease of CAIX mRNA levels ($p<0.001$). PHD2 and PHD3, which regulate HIFα stability in an oxygen-dependent manner, are HIF target genes themselves (Metzen *et al.* 2005; Pescador *et al.* 2005). A similar regulation as for CAIX by 5 nM 17-AAG was seen for PHD3 mRNA but not PHD2 mRNA (Fig. 5). PHD1 mRNA was barely detectable in these cells (data not shown). Previously, it has been reported that PHD3 is strongly induced by hypoxia, which is mediated by HIF-1 (Pescador *et al.* 2005). The hypoxic induction of PHD3 limits HIF-1α accumulation during long time hypoxia and enables rapid destabilization following reoxygenation (Appelhoff *et al.* 2004; Marxsen *et al.* 2004). The increased expression of PHD3 by hypoxia and 5 nM 17-AAG may explain the downregulation of hypoxic stabilization of HIF-1α after 24 hours as well as the diminished effect of 5 nM 17-AAG after 24 hours treatment.

2.4.4.5 Effects of HSP90 inhibitors on angiogenesis

Besides metabolic tumor adaptation towards hypoxia, tumor angiogenesis is a major event during tumor progresssion. A major regulator of tumor angiogenesis is the HIF-1 downstream target VEGF. Therefore, we investigated whether HSP90 inhibitors also induce VEGF synthesis. Consistent with the reporter and target gene studies, treatment of HeLa or Hep3B cells with 5 nM 17-AAG increased hypoxia-induced VEGF secretion, whereas treatment with 3 μM significantly reduced VEGF production (Fig. 6A and B).

In two previous studies the effects of GA, 17-AAG or 17-DMAG on angiogenesis were investigated in Matrigel angiogenesis assays (Kaur *et al.* 2004; Sun and Liao 2004). In both studies HSP90 inhibitors decreased the motility response of endothelial cells to VEGF or fibroblast growth factor-2 in a dose-dependent manner. However, in a complex tumor model the effects of HSP90 inhibitors not only on endothelial cells but also on tumor cells have to be taken into account. Our data suggest that HSP90 inhibitors may increase or decrease the HIF-system, including the expression of VEGF in tumor cells, depending on the local concentrations. VEGF acts on endothelial cells as both chemotactic and mitogenic factor, via cell-specific receptors. VEGF within a solid tumor is produced in part by endothelial cells, in part by tumor cells, in part by stroma cells and in part by invading macrophages, mainly as a result of changes in oxygenation (Tang *et al.* 2004). In order to mimic the complexity of the *in vivo* situation, we thus used the CAM assay to investigate the effect of HSP90 inhibitors on angiogenesis. Treating CAMs with 5 nM and 3 μM 17-AAG resulted in significant changes compared to the solvent-treated control CAMs. In line with HIF-1 transcriptional activity and VEGF production, 36% (5 out of 14 total) of 5 nM 17-AAG-treated eggs showed clear signs of pro-angiogensis and 50% (5 out of 10 total) of 3 μM 17-AAG-treated eggs showed signs of deteriorated and decreased vessel formation as illustrated in Fig. 6B. None of the eggs treated with 5 nM or 3 μM 17-AAG showed signs of anti-angiogenesis or pro-angiogenesis, respectively.

Taken collectively, our data demonstrate bimodal effects of HSP90 inhibitors on the HIF-system which are highly dose-dependent. HIF-1α stabilizing

effects have also been described for other anti-cancer/chemopreventive agents like epicatechin gallate and cisplatin (Yang *et al.* 2004; Zhou *et al.* 2004). These data support the need for the development of more specific inhibitors of the HIF-system since the *in vivo* effects of pleiotropic HSP90 inhibitors are difficult to predict over a wide dose-range.

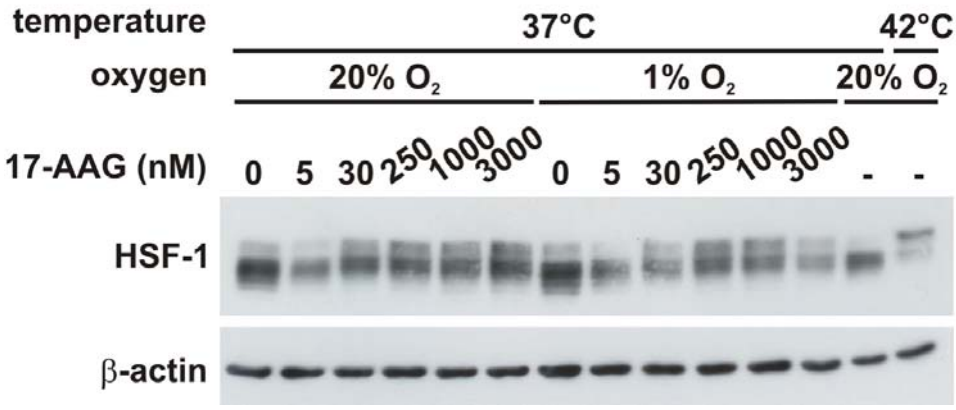

Figure 1. 17-AAG activates a heat shock response in HeLa cells. HeLacells were treated under normoxic (20% O_2) or hypoxic (1% O_2) conditions with increasing concentrations of 17-AAG as indicated. After 4 hours, the cells were lysed and HSF-1 activation by phosphorylation was detected by immunoblotting as described in Material and Methods. Protein extracts derived from HeLa cells incubated at 37°C or 42°C for 4 hours were used as negative and positive controls, respectively, for HSF-1 activation. As a control for equal loading and blotting, β-actin was subsequently determined on the same blot.

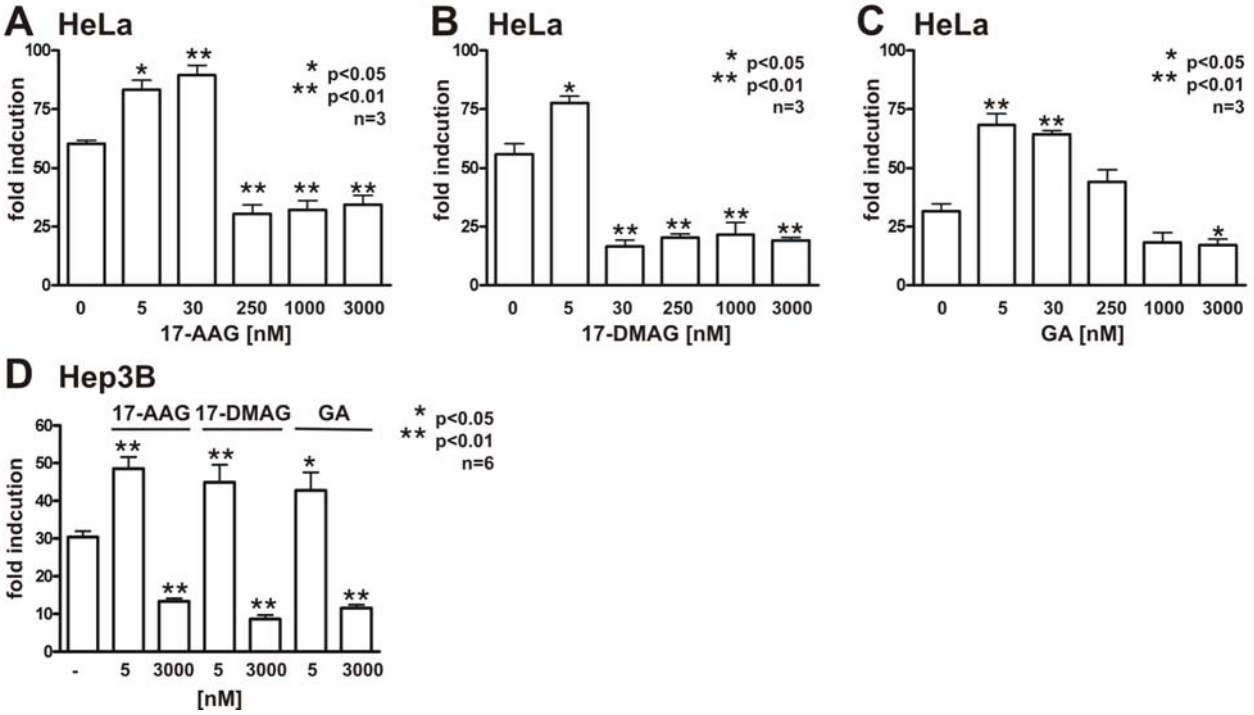

Figure 2. HSP90 inhibitors affect HIF-1 activity. (A-C) HeLa cells were transiently transfected with a HIF-1-dependent reporter gene plasmid as described in Materials and Methods. Subsequently, the cells were treated with the indicated concentrations of 17-AAG (A), 17-DMAG (B) or GA (C) and exposed to 20% O2 or 1% O2. After 24 hours, the cells were lysed and luciferase activity was measured. Induction factors were normalized to non-treated cells cultured under normoxic conditions. Mean values ± SEM of n = 3 independent experiments are shown. (D) Reporter gene assays were performed in Hep3B cells as described above. The concentrations of 17-AAG, 17-DMAG or GA are indicated. Mean values ± SEM of n = 6 independent experiments are shown.

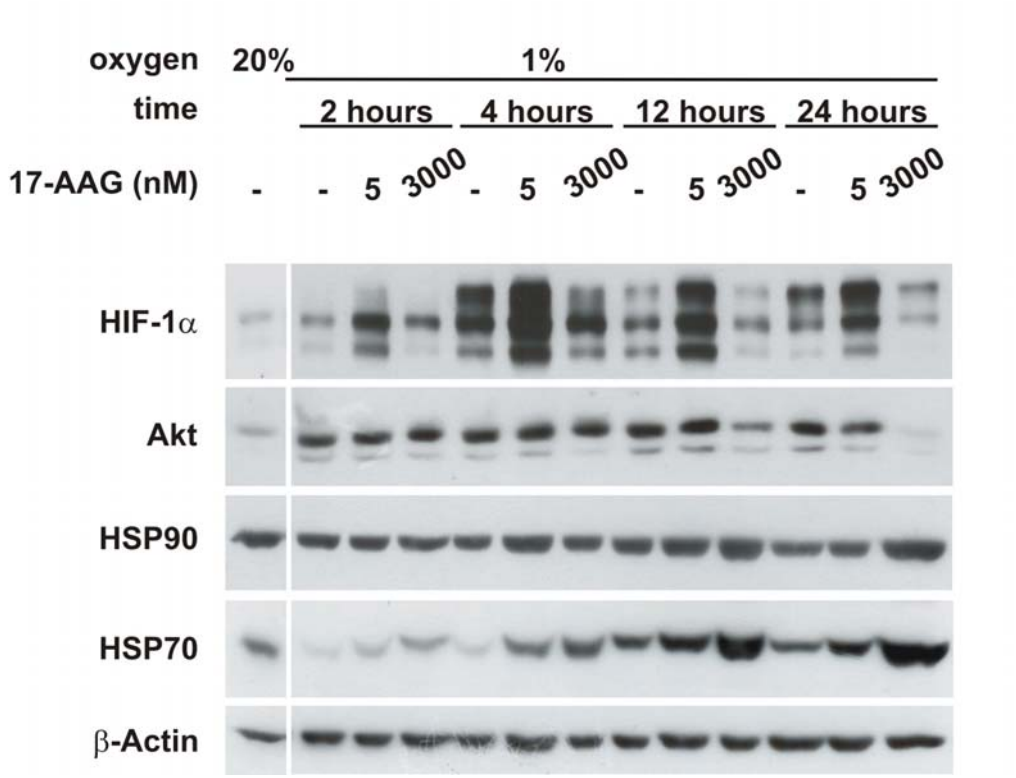

Figure 3. Effects of HSP90 inhibitors on HIF-1α protein levels. HeLa cells were treated for 2-24 hours with 17-AAG at the indicated concentrations under hypoxic (1% O2) conditions. Subsequently, combined cytoplasmic and nuclear protein was extracted and HIF-1α, Akt, HSP70, HSP90, and β-Actin were detected by immunoblotting.

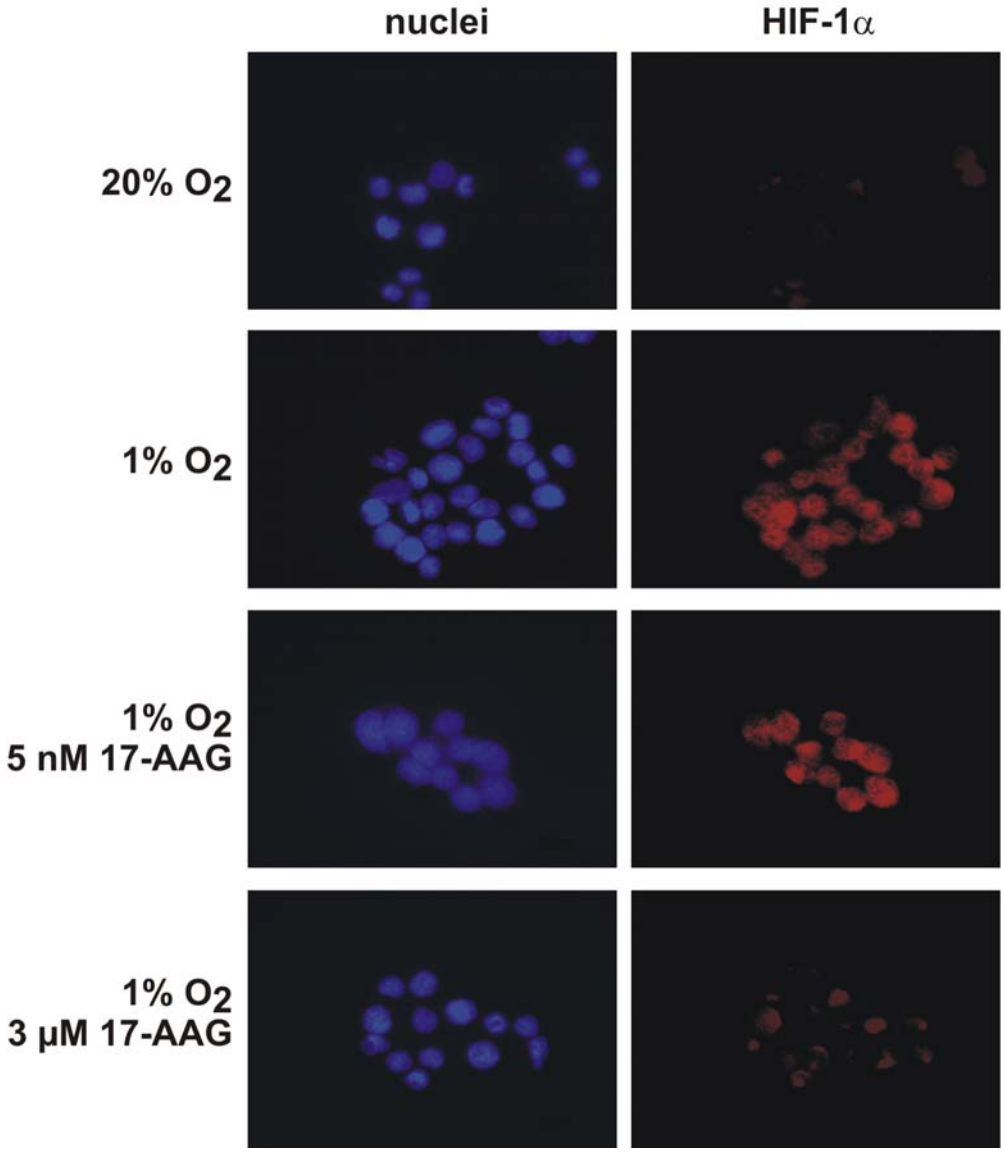

Figure 4. Subcellular localization of HIF-1α after treatment with 17-AAG. HeLa cells were exposed to normoxic (20% O2) or hypoxic (1% O2) conditions without or with the addition of 5 nM or 3 μM 17-AAG for 4 hours. The cells were prepared for indirect immunofluorescence analysis as described in Materials and Methods. Left panel, Hoechst 33258 staining of the nuclei; right panel, anti-HIF-1α staining using a TRITC-coupled secondary antibody.

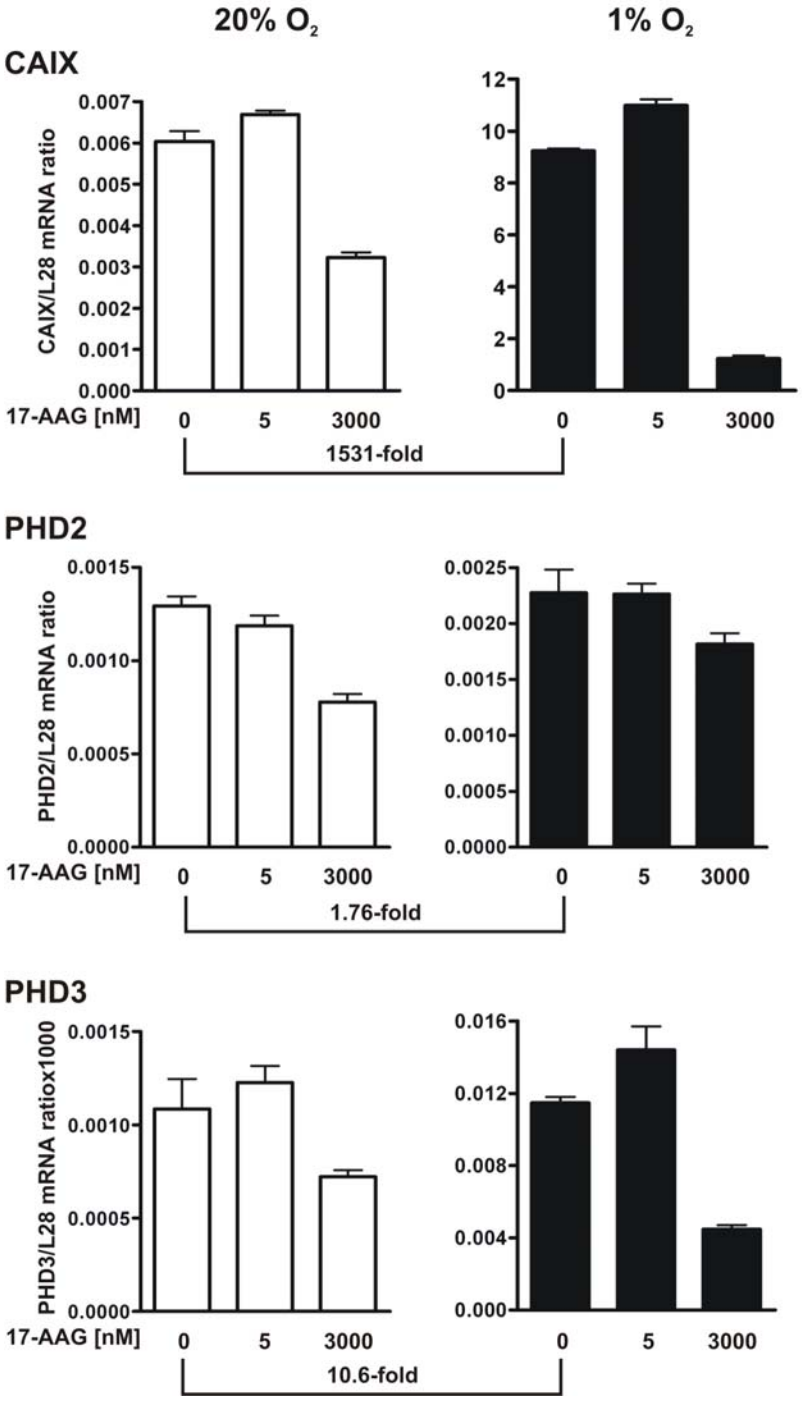

Figure 5. Effects of 17-AAG on HIF-1 target gene expression. Hep3B cells were exposed to normoxic (20% O2) or hypoxic (1% O2) conditions without or with the addition of 5 nM or 3 μM 17-AAG for 24 hours. The cells were lysed, total RNA was extracted, reverse transcribed and the CAIX, PHD2, PHD3 and L28 mRNA levels were quantitated by real-time PCR. Mean values ± SEM of n = 3 are shown.

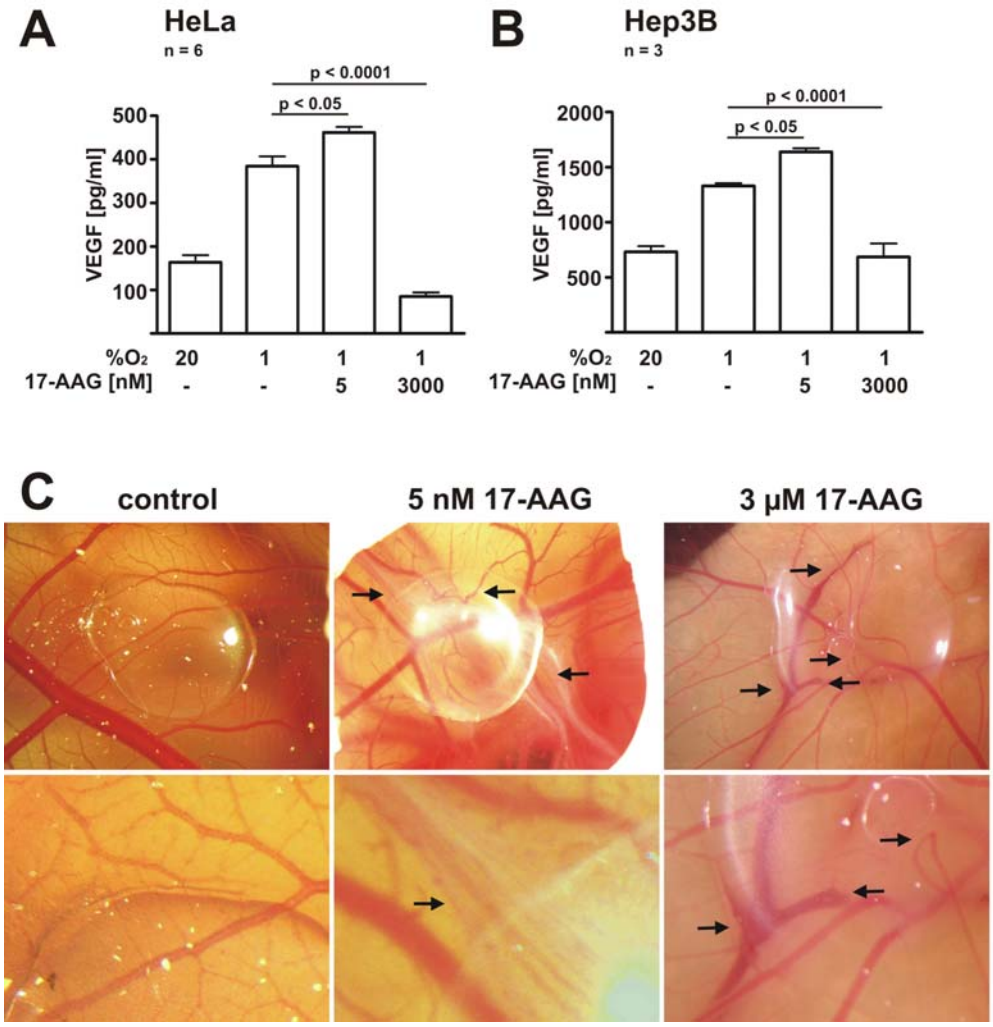

Figure 6. Effects of 17-AAG on VEGF protein secretion and angiogenesis in the CAM. (A) HeLa cells or (B) Hep3B cells were exposed to normoxic (20% O2) or hypoxic (1% O2) conditions for 24 hours without or with the addition of 5 nM or 3 μM 17-AAG. Subsequently, the supernatants were collected and VEGF protein concentration was estimated by ELISA. Mean values ± SEM of n = 6 experiments (HeLa cells) or n = 3 experiments (Hep3B cells) are shown (C) Inert polymer discs containing solvent only (control), 5 nM or 3 μM 17-AAG were placed onto the CAM at day 10 of embryonic development as described in Materials and Methods. Pictures were taken three days later.

2.5 Unpublished data

2.5.1 Introduction

DNA-PKcs mRNA level in wild-type MEF-*Hif1a*$^{+/+}$ are significantly higher than in mutant MEF-*Hif1a*$^{-/-}$, possibly resulting in a different etoposide sensitivity of the cell lines. We tried to further elucidate the HIF-dependent nature of the observed difference. However, stable as well as transient reconstitution of the mutant MEF-*Hif1a*$^{-/-}$ cell line resulted in no detectable HIF target gene induction upon hypoxic exposure. Therefore other strategies should reveal the mechanism behind the observed etoposide resistance of mutant MEF-*Hif1a*$^{-/-}$.

In a first approach we established stable shRNA clones of the wild-type MEF-*Hif1a*$^{+/+}$. Two clones (Bd10, Be7) with stably down-regulated HIF (Fig. 1A and 1C) were obtained by clonal selection for cells having only marginal HIF-dependent PHD3 (one of the strongest induced genes by HIF) induction (Fig. 1B). In order to have a control, one additional clone (Bc12) with intact PHD3 induction in hypoxia was chosen (Fig. 1B).

However, none of the three shRNA transfected wild-type MEF-*Hif1a*$^{+/+}$ clones differed in their sensitivity towards etoposide when compared to each other and to the wild-type cell line MEF-*Hif1a*$^{+/+}$ (Fig. 1D). Even though HIF-dependent target gene induction, as expected, is blunted in the clones Bd10 and Be7 they did not show any sign of enhanced etoposide sensitivity.

In addition, also known HIF inhibitors (Fig.2) could not sensitize wild-type MEF-*Hif1a*$^{+/+}$ to etoposide (Fig. 3). Thus, the observed etoposide sensitivity might require only minimal basic levels of HIFα to become apparent and may be independent of HIFs transcriptional activity as indicated by the missing effect on etopside sensitivity upon hypoxic HIF induction or the inhibition of HIF by chemical compounds.

Indeed, reporter gene assays with the mouse or human DNA-PKcs promoters could not provide any hint to a transcriptional activity directed by HIF. On the contrary, the over-expression of HIFα in HeLa cells did not activate the DNA-PKcs promoter construct (Fig. 4), neither did HIFα, transiently introduced into mutant MEF-*Hif1a*$^{-/-}$ cells, result in an enhanced

expression of luciferase from the DNA-PKcs reporter construct when compared to a transfected β-galactosidase expression vector as a negative control (Fig. 5). However, transcriptional activity of the DNA-PKcs promoter is different when comparing the MEF cell lines wild-type MEF-*Hif1a*$^{+/+}$ and mutant MEF-*Hif1a*$^{-/-}$, providing evidence for another factor, affected by the absence of HIFα, that influences transcription control of DNAPKcs.

2.5.2 Materials and Methods

2.5.2.1 Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 4'-desmethylepipodophyllotoxin 9-(4,6-O-ethylidene-b-D-glucopyranoside) (etoposide) were purchased from Sigma.

17-dimethylaminoethylamino-17-demethoxygeldanamycin (DMAG), 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) were a kind gift of Kosan Biosciences Inc. (Hayward, CA). (5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC1) was purchased from Biomol international, L.P. (Plymouth Meeting, PA) All three drugs were dissolved in DMSO to generate 10 mM stock solutions. All further dilutions were prepared in cell culture medium.

2.5.2.2 Cell culture

MEFs were derived from mouse day 9.5 embryos, either wild-type (MEF-*Hif1a*$^{+/+}$) or deficient (MEF-*Hif1a*$^{-/-}$) for HIF-1α, immortalized with SV40 large T antigen and transformed with H-*ras* (Ryan *et al.* 2000; Seagroves *et al.* 2001). All cell lines were cultured in Dulbecco's modified Eagle's medium (high glucose) as described previously (Stiehl *et al.* 2006). For hypoxic experiments, MEFs were grown in a gas-controlled glove box to handle the cells under constant oxygen (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK).

2.5.2.3 Protein extractions and immunoblot analyses

Cells were washed twice and scraped into ice-cold PBS. Soluble nuclear protein was extracted with a high-salt extraction buffer containing 0.1% NP-40 essentially as described before (Stiehl et al. JBC 2006). Protein concentrations were determined by the Bradford method and 80 μg protein

was subjected to immunoblot analysis. Primary antibodies for HIF-1α was from Novus Biologicals (Littleton, CO). Bound antibodies were detected by respective secondary antibodies (Pierce) and visualized with ECL substrate (Pierce).

2.5.2.4 mRNA quantification

Total RNA was extracted as described previously (Stiehl *et al.* 2006). Following reverse transcription (RT) of 5 μg total RNA, mRNA levels were quantified with 1% of diluted cDNA reaction by real-time RT-PCR. A SybrGreen qPCR reagent kit (Sigma) was used in combination with a MX3000P light cycler (Stratagene). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein S12 mRNA was determined and data expressed as ratios relative to S12 levels. Melting point analyses of amplified PCR products were performed after each run to verify specific amplification.

2.5.2.5 MTT cell viability assay

Cells were plated onto 96-well plates and allowed to attach o.n. previous to exposure to various concentration of etoposide and/or HIF-1α inhibitors (17-AAG, DMAG, YC-1). Following exposure for 24h or 48h 10 μl MTT (5 mg/ml) was added for up to 12 h and incubated under 37°C, 5% CO_2 atmosphere. Solubilization of the formed formazan crystals with 100 μl 10% sodium dodecyl sulphate (SDS), 0.001 M HCl for 24 h resulted in yellow to purple colored wells. Subsequent determination of absorbance at 550 nm relative to a wavelength of 630 nm was performed with a microplate reader.

2.5.2.6 Generation of HIF-1α shRNA MEF cell lines

MEFs wild-type (MEF-*Hif1a*$^{+/+}$) or mutant (MEF-*Hif1a*$^{-/-}$) were transfected using PEI with a pSilencer 2.6 vector (Ambion, Huntington, United Kingdom) containing a modified HIF-1α sequence. The original sequence has been shown to effectively down regulate HIF-1α (Erler *et al*. 2004) and was modified to 5`GTCTCGAGATGCAGCCAGA-3` corresponding to the

respective murine mRNA sequence. Cells were selected for stable transfection using hygromycin-containing (300 μg/μl) medium. Stably expressing clones were obtained by diluting the transfected pool and plating onto 96-well plates. Immunoblot for HIF-1α, mRNA level of HIF-1α and induction values of HIF-1α target genes was used to validate stable expresson of HIF-1α shRNA.

2.5.2.7 Plasmids

DNA isolated from MEFs wild-type (MEF-*Hif1a*$^{+/+}$) or mutant (MEF-*Hif1a*$^{-/-}$) was used to amplify the promoter region of DNA-PKcs by PCR. Luciferase reporter constructs were obtained by cloning a fragment corresponding to the DNA-PKcs promoter into the pGl3-basic vector. In detail, the region from –719 to +1 flanked NheI and XhoI was inserted into pGL3-basic leading to pGL3_mPRKDC. The vectors pGL3_hPRKDC was a kind gift from (CW. Anderson). Construction of the pH3SVL, containing a total of 6 HIF-1 DNA-binding sites derived from the transferrin gene by calcium phosphate co-precipitation method as described previously (Rolfs *et al.* 1997). Co-transfected renilla luciferase control plasmid pRL-SV40 (Promega, Mannheim, Germany) served to normalize transfected DNA content.

2.5.2.8 Transfection and luciferase assay

Transient transfection was performed on 100 mm dishes using PEI. To equalize the transfected DNA amount pcDNA3 or pcDNA3_CMV was used. In order to normalize for transfection efficiency a renilla reporter gene was co-transfected. Transfected cells were grown over night and split onto 6-well plates priory to normoxic or hypoxic (16 h, 0.2% O_2) incubation. Cell lysates were prepared with passive lysis buffer (Promega) and aliquots were assayed for luciferase expression with a microplate luminometer (Berthold, Regensdorf, Switzerland).

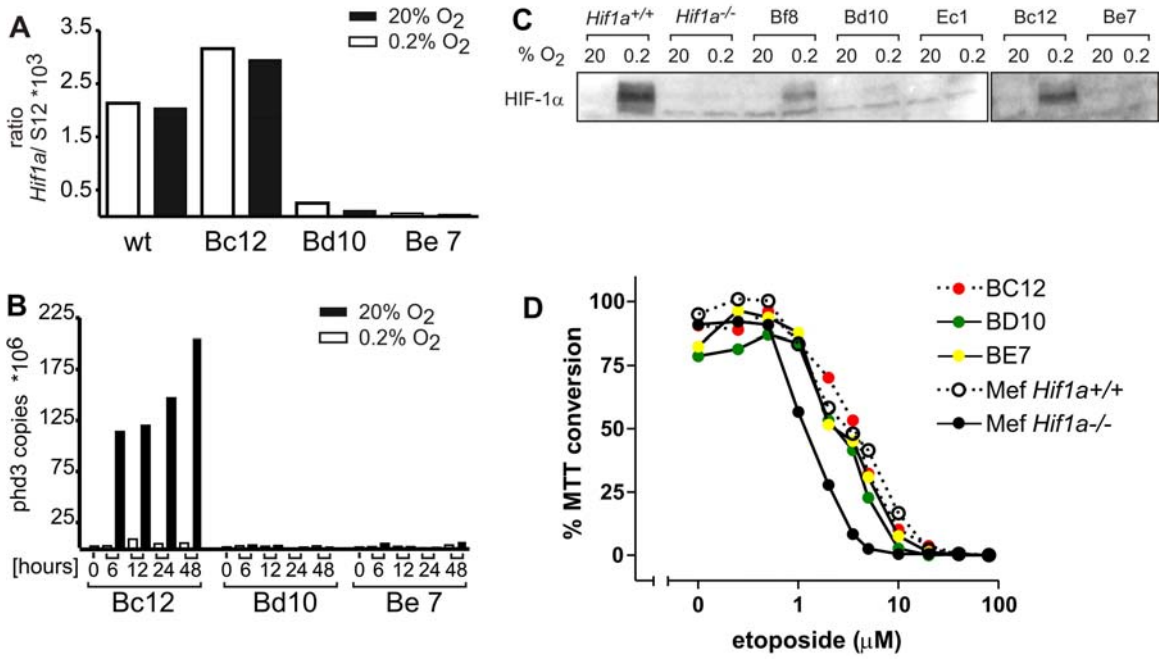

Figure 1. HIF-1α-shRNA stably down-regulated clones of the MEF wild-type (*Hif1a*$^{+/+}$) cell line.

(A) Normalized HIF-1α mRNA levels as determined by quantitative real-time PCR. MEF wild-type (*Hif1a*$^{+/+}$) and selected HIF-1α-shRNA stable down-regulated clones (Bc12 with intact HIF-1α levels; Bd10, Be7 with suppressed HIF-1α levels). (B) Normalized mRNA levels of a HIF-1α target gene (PHD3) as determined by RT-qPCR. Bc12 with intact hypoxic response, Bd10 and Be7 without hypoxic HIF-1α target gene activation. (C) Immuno-detection of HIF-1α-in wild-type (*Hif1a*$^{+/+}$) and mutant (*Hif1a*$^{-/-}$) cells and HIF-1α-shRNA stable down-regulated clones (termed Bf8, Bd10, Ec1, Bc12, Be7). (D) Etoposide sensitivity of the HIF-1α-shRNA stably down-regulated clones (Bc12, Bd10, Be7), MEF wild-type (*Hif1a*$^{+/+}$) and MEF mutant (*Hif1a*$^{-/-}$). Only the mutant (*Hif1a*$^{-/-}$) cells differed in their sensitivity towards etoposide.

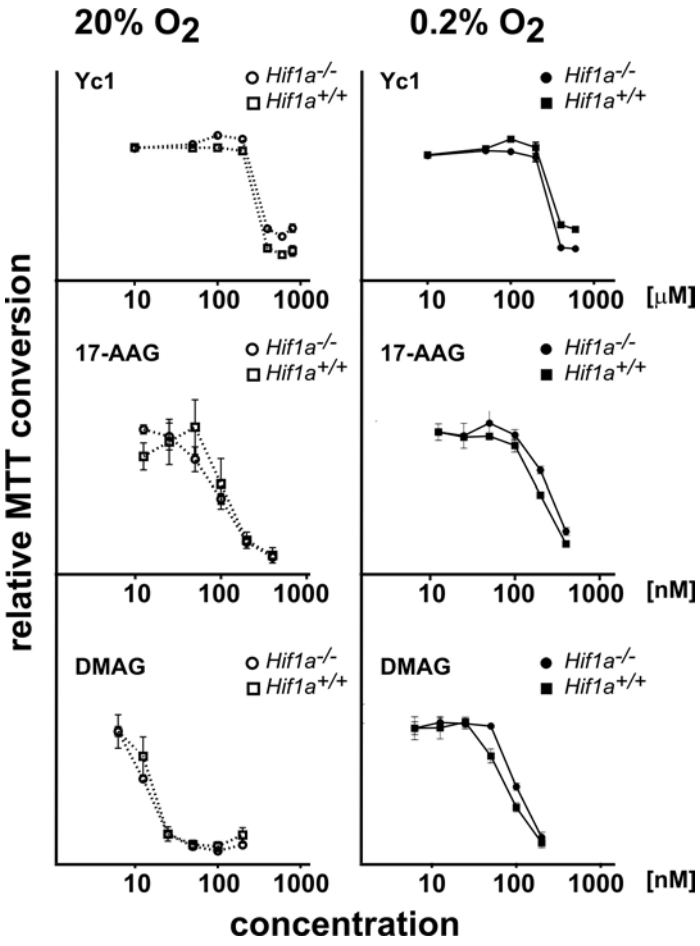

Figure 2. Titration curve of HIF inhibitors in MEF wild-type (*Hif1a*$^{+/+}$) and MEF mutant (*Hif1a*$^{-/-}$). Cells were exposed to the drugs for 24h and MTT assay was used to quantify the amount of viable cells (that is toxicity of the compounds). Under normoxic (20% O_2) as well as under hypoxic (0.2% O_2) conditions the inhibitors were equally toxic for both cell lines MEF wild-type (*Hif1a*$^{+/+}$) and MEF mutant (*Hif1a*$^{-/-}$). However, under hypoxia higher doses of the ansamycin derivatives (17-AAG, DMAG) had to be applied when compared to normoxia, even though the concentrations were identically higher in the two ell lines MEF wild-type (*Hif1a*$^{+/+}$) and MEF mutant (*Hif1a*$^{-/-}$).

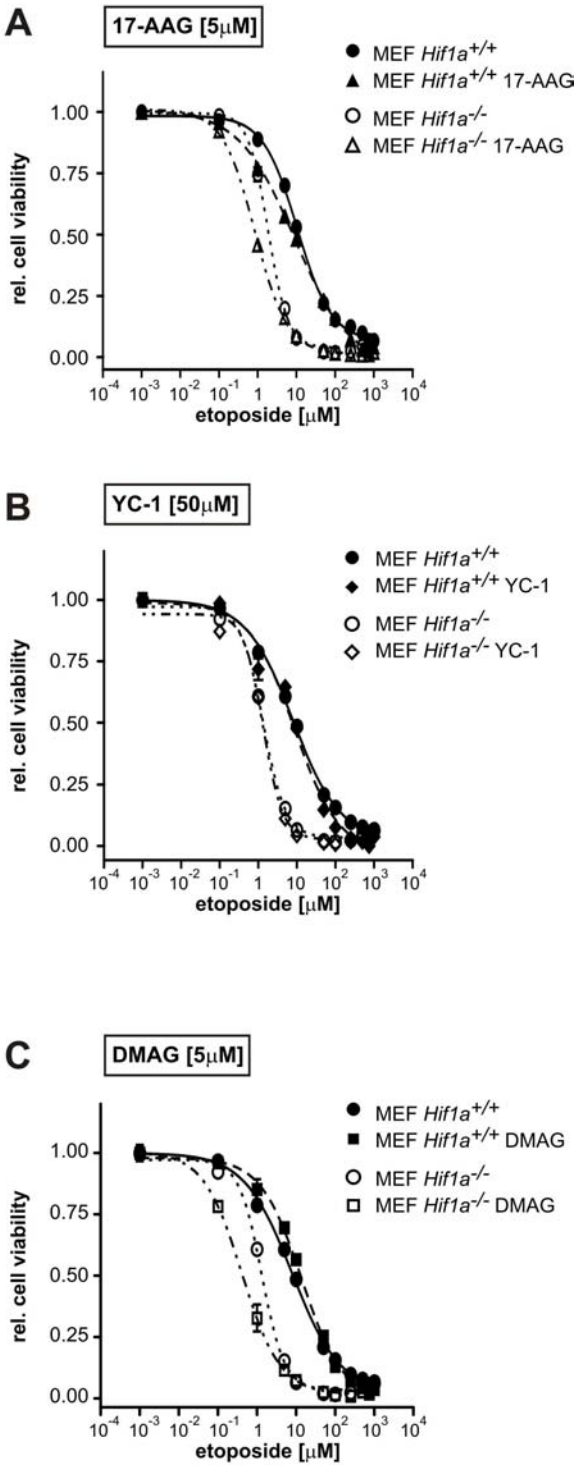

Figure 3. (A) Titration of etoposide to wild-type (*Hif1a*$^{+/+}$) and MEF mutant (*Hif1a*$^{-/-}$). in the presence or absence of 17-AAG. ATP concentration were measured by determining luciferase activity, thus reflecting cell viability. 24 h exposure to etoposide at different concentrations. The cells wild-type MEF (*Hif1a*$^{+/+}$) and mutant MEF (*Hif1a*$^{-/-}$) differs in their sensitivity towards etoposide. Addition of 17-AAG (5 μM) had no effect. Data points were measured as triplicates.

(B) Titration of etoposide to wild-type (*Hif1a*$^{+/+}$) and MEF mutant (*Hif1a*$^{-/-}$) in the presence or absence of YC-1. MTT conversion capacity was used to determine the amount of viable cells after 24 h exposure to etoposide at different concentrations. The cells wild-type MEF (*Hif1a*$^{+/+}$) and mutant MEF (*Hif1a*$^{-/-}$) differs in their sensitivity towards etoposide. Addition of YC1 (50 μM) had no effect. Data points were measured as duplicates.

(C) Titration of etoposide to wild-type (*Hif1a*$^{+/+}$) and MEF mutant (*Hif1a*$^{-/-}$) in the presence or absence of DMAG. MTT conversion capacity was used to determine the amount of viable cells after 24h exposure to etoposide at different concentrations. The cells wild-type MEF (*Hif1a*$^{+/+}$) and mutant MEF (*Hif1a*$^{-/-}$) differs in their sensitivity towards etoposide. Addition of DMAG (5 μM) had no effect. Data points were measured as duplicates.

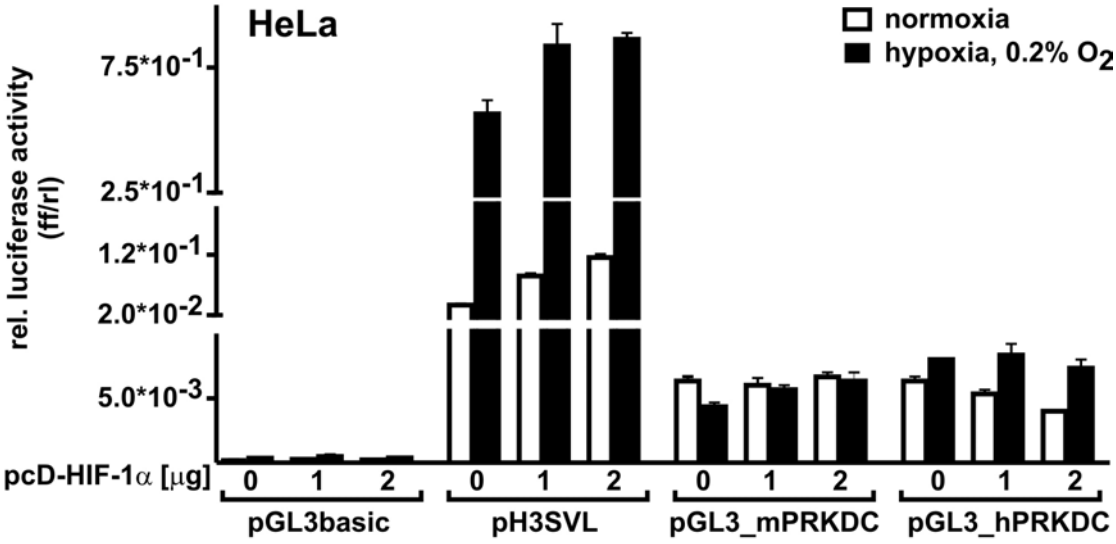

Figure 4. Titration of HIF-1α in HeLa cells in combination with luciferase reporters. Co-transfection of a mouse or human DNA-PKcs promoter construct with a plasmid expressing HIF-1α did not induce luciferase expression. pH3SVL as positive control (with 3 hypoxia response elements (HRE)) could be activated to express luciferase by addition of HIF-1α under normoxia and hypoxia. The empty vector did express only basaic levels of luciferase.

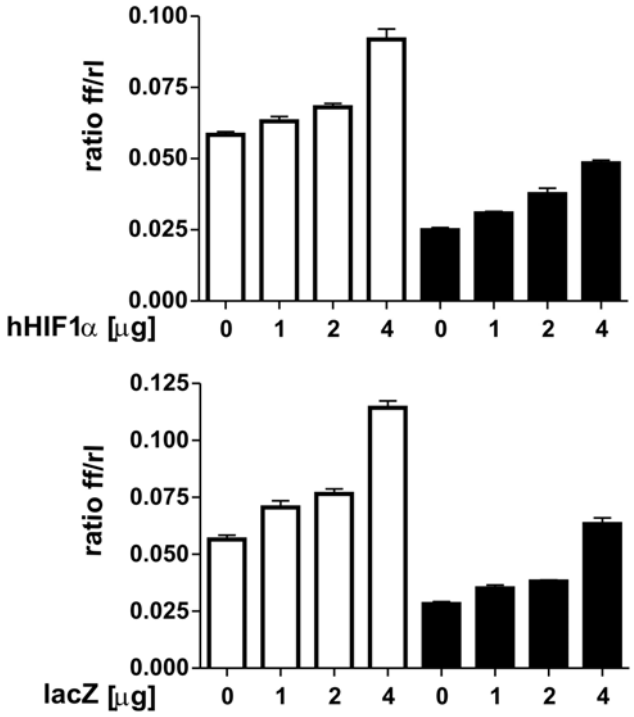

Figure 5. Titration of HIF-1α mutant MEF-*Hif1a*$^{-/-}$ cells. Transfection of increasing amounts of a HIF-1α expressing plasmid resulted in minimal enhanced expression from the DNA-PKcs co-transfected reporter construct (4 μg) under normoxia (white bars) as well as under hypoxia (black bars). However, same trend was observed with a lacZ plasmid expressing β-galactosidase. Thus, no HIF-1α specific effect on the DNA-PKcs promoter could be observed.

3 Discussion

3.1 Oxygen sensing

3.1.1 Acute and chronic oxygen-dependent regulation of HIF

In order to respond to changes in the oxygen partial pressure a, cell has to register the available oxygen concentrations which is then translated by the transcription factor HIF in the respective transcriptional profile as part of the cell`s response. The “sensory system” consists of PHDs which depend on molecular oxygen for their function. Therefore oxygen concentration is closely linked to PHD activity. On the other hand, the PHDs are responsible for the degradation of HIFα by adding oxygen to a proline residue in the ODD domain. Thus, PHD activity is also linked to the concentration of HIF, which in turn results in the transcriptional profile of the cellular response to changes in oxygenation. This mechanism hence provides a cell with an ideally suited switch to trigger the activation of genes necessary to endure periods of acute hypoxia. However, it might also be possible that basic levels of HIFα are permanently adjusted to the prevalent concentration of oxygen by the same system under chronic hypoxic conditions.

3.1.2 Prolyl-4-hydroxylases set the basic levels of HIFα under chronic hypoxia

The concentration of HIFα is not permanently up-regulated during hypoxic exposure. After a short period of time with elevated HIFα-levels, reflecting an acute response to the hypoxic stimulus, HIFα-levels decrease and settle at a different set point; possibly corresponding to the current oxygen concentration of the chronically hypoxic conditions. In addition, HIF target genes, after being induced, decrease to permanently higher levels than before exposition to hypoxia reflecting a similar response as HIFα protein. Even though the oxygen sensory system, i.e. the PHDs, was characterized for its behavior under standard conditions of acute hypoxia, providing evidence for a linear HIFα regulation over the whole range of physiologically relevant oxygen

concentrations, the role of HIFα regulation under chronic hypoxia by the PHDs is not well understood. During tumor formation, cancerous areas are exposed to acute as well as to chronic hypoxia. The connection between PHD activity and the (re)setting of basic levels of HIFα under chronic hypoxic conditions might impact on tumor progression and tumor therapy outcome.
During *in vitro* VHL-binding assays we could show that PHD activity remains detectable even under severe hypoxic conditions, when oxygen as substrate is limited, thus representing the rate determining component in the processes leading to the degradation of HIFα. Also degradation of a HIFα-ODD domain containing reporter gene constructs occurred efficiently under severe hypoxia. Thus, PHDs retain significant hydroxylase activity even under severe hypoxic conditions. Furthermore, PHD2 and PHD3 mRNAs are HIF-dependently induced. This results in increased concentrations of PHD2 and PHD3 proteins, strictly depending on the concentration of HIFα and HIF activity respectively. Since increased concnetrations of an enzyme is equivalent to increased activity under otherwise unchanged conditions, PHD2 and PHD3 in concert with HIFα thus might establish a feedback loop that levels off the concentration of HIFα during chronic hypoxia. Of note, induced PHD2 and PHD3 protein levels in effect correlate nicely with decreased HIFα, indicating interweaved relations of sensory and processor system.

3.1.3 Prolyl-4-hydroxylase assays

In light of the strong link between HIF activity and PHD activity it appears probable that the correlation between elevated HIF and tumor therapy resistance might indeed be caused by the PHD system, potentially involving other factors regulated by the PHDs. There are in fact several proteins interacting for each PHD variant and at least two transcription factors (ATF4) was suggested to be hydroxylated by PHD3 in addition to HIF-1α (Koditz *et al.* 2007). Therefore we established PHD assays to measure hydroxylation of potentially new PHD targets. One methode we used to detect hydroxylation was in-gel digestion followed by mass spectrometry (ms). However, *in vitro* hydroxylation did not yet result in a percentage of hydroxylated protein high enough to be detected by ms.

An other method to determine hydroxylation is to monitor oxygen addition by targeting the hydroxylated moiety with a specific interactor. The VHL-binding assay is based on the specific recognition of the hydroxylated HIFα -ODD domain by pVHL. We used this method to quantify hydroxylation of HIFα -ODD domain under various experimental conditions. However, due to its specificity this assay is purely restricted to the ODD domain of HIFα, thus not adaptable to unknown proteins.

A completely different approach makes use of the co-substrate conversion by the PHDs to measure PHD activity. We exploited the conversion of radioactively labeled oxoglutarate to radioactively labeled succinate to determine PHD activity. Unfortunately, when using crude protein extracts as source of PHD activity the reaction of oxoglutarate to succinate is also performed by other enzymes, resulting in a very high background and impeding a clear quantification of the activity of the PHDs. Thus purification of the target protein as well as the PHD is required for this assay. In addition, the reaction is stoichiometrical; meaning that per hydroxylation event only one oxoglutarate will be converted to succinate. When using large proteins it is difficult to obtain enough concentrated protein solutions to provide sufficient hydroxylation sites and thus enough conversion reactions of oxoglutarate to succinate in order to pass a minimal threshold of succinate formed. Neverthless, the application of this assay to discover hydroxylation of novel PHD substrates should be possible, eventually leading to the identification of novel, only “indirectly” HIF-dependent factors involved in tumor therapy resistance.

3.2 HIF and cancer therapy resistance

3.2.1 HIF and cancer progression: a positive factor in tumor growth

High HIF levels correlate with tumor progression and tumor therapy resistance.(Unruh *et al.* 2003; Moeller and Dewhirst 2006) The contribution of HIF to cancer progression seems to depend on the tumor micro-environment and to a major part on its involvement in vasculogenesis.(Pouyssegur *et al.* 2006) The tumor environment selects for tumor cell phenotypes and cells with

(pathologically) up-regulated HIF levels profit from the set of HIF-target genes.(Semenza 2003; Wenger *et al.* 2005) The HIF-dependent release of cytokines and growth factors build the base for further proliferation of the cancerous lesions. Thus, HIF will sustain cellular life and trigger the formation of new blood vessels. Vascularization is crucial for the tumor mass to grow over a limited size, underlining the importance of HIF in the course of cancer development. (Hanahan and Weinberg 2000; Weinberg 2007)

3.2.2 HIF and cancer therapy: a negative factor in tumor therapy

Tumor progression and tumor therapy are connected since faster growing and more voluminous tumors are more difficult to treat. The tumor growth-promoting effect of HIF (due to its central role in angiogenesis) would already be sufficient to target the transcription factor as a negative factor in tumor therapy. However, several studies attributed additional functions to HIF in tumor therapy; independent of angiogenesis. Cell culture studies with cell lines deficient for HIF or impaired in HIF function (dominant negative mutants, molecular inhibitors, or RNAi studies) confirm on a molecular action exerted by HIF, which shelter cells from chemo- or radio-therapeutic treatments. (Unruh *et al.* 2003; Erler *et al.* 2004; Zhang *et al.* 2004; Williams *et al.* 2005; Brown *et al.* 2006; Hussein *et al.* 2006; Song *et al.* 2006) However, the interpretation of the observed therapy resistance differs and no definitive answer to HIF`s role in therapy resistance could be given up to date.

3.2.3 Studying HIF in vitro; the cell culture model

We addressed the question of HIF`s contribution to therapy resistance by the use of mouse embryonic fibroblast cell lines, either wild-type MEF-*Hif1a*$^{+/+}$ or mutant MEF-*Hif1a*$^{-/-}$. In half of all human cancer cell genomes p53 is mutated and p53 signaling networks are probably altered in every malignant transformation. (Weinberg 2007) In order to mimic cancer cell conditions p53 was inactivated by SV40 largeT-antigen introduction, leading to immortalization. Moreover, transformation with *HRAS* to enhance proliferation rate and cancer cell behavior was performed. (Ryan *et al.* 2000)The use of embryonic fibroblasts was believed to result in enhanced homogeneity of the transcriptional profile (as compared to embryonic stem cells). During all

experiments high glucose concentration was used to keep intracellular ATP constant and thus be independent of metabolic effects unrelated to HIF.

3.2.4 Different behavior of the cell lines after drug administration

By comparing the two cell lines for their sensitivity to known genotoxic agents such as etoposide a clear difference in cell viability was observed. Likewise tumor-regression in xenografted animal models was higher in case of the mutant MEF-*Hif1a*$^{-/-}$ cells after etoposide administration. Puzzeling, however, the cell lines showed the same difference on etoposide treatment under normoxic conditions as well as under hypoxic conditions. Normoxia is used to describe the availability of oxygen in air at sea level atmospheric pressure, thus refers to the oxygen partial pressure (pO_2) of room air. During cell culture studies "normoxic" experiments are operated with 18% oxygen in the gas phase (due to the use of a gas mixture containing nitrogen, carbon dioxide (5%) and oxygen - the room air pO_2 (160 mmHg) corresponds to 18% oxygen in this gas mixture). Hypoxic experiments on the other hand specify conditions with lower percentages of oxygen in the gas mixture, thus with a lowered pO_2. By decreasing the pO_2 in cell culture experiments HIF gradually becomes stabilized until at mild hypoxia (at around 5% oxygen (pO_2 of around 40 mmHg)) HIF is detectable by immunoblotting with maximal levels at around 0.2% oxygen (pO_2 of 1.6 mmHg). Therefore, HIF accumulation (that is hypoxic incubation vs. normoxic cultivation of the cell line) had no effect on the displayed etoposide sensitivity, which obviously not responds to acute (*viz*. inducible) HIF activity. Furthermore, under normoxic conditions HIF target gene expression is marginal due to the degradation of HIF and the inhibitory modification by FIH. But how could the observed resistance be assigned to HIF?

3.2.5 HIFα stabilization in the cell culture model system

When comparing HIFα levels of normal healthy tissue with neoplastic tissue, elevated HIFα levels are detected in the malignant lesion in the majority of the cases. HIFα is not only controlled by the PHDs. In addition, activated MAPK pathways contribute to the action of HIFα and the PI3K pathway also plays a

role in the regulation of the activity of the transcription factor. (Jiang *et al.* 2001) Both pathways converge in the up-regulation of general protein synthesis by stimulation of protein translation. In the case of HIFα the fine-tuned turn-over of synthesis and degradation becomes easily disturbed. While HIFα-degradation continues at same pace the rate of protein synthesis is elevated and thereby HIFα accumulates. However, at present still inhibited by FIH hydroxylation. Both pathways are commonly up-regulated in cancer and are activated in the cell culture model system used in this study. Thus, it seems very likely that HIF-dependent effects can be found under normoxic conditions owing to the elevated HIFα levels in cancer tissue and probably also in the cell culture model system. Under normoxic cell culture conditions HIFα may indeed have a transcriptional function as indicated by the observation that, even cultured under normoxia, HIFα-deficient cells have a reduced gene expression of HIF-1 target genes.

3.2.6 HIF function unrelated to the available oxygen amount - "oxic" activity of the N-terminal trans-activation domain

The nature of the transcription factor HIF appears to be quite distinct from the nature of other transcription factors. Not only the mode of regulation, with HIFα subunits being permanently degraded, but also the fact that HIFα contains two trans-activation domains separates it from the bulk of other transcription factors. HIF target gene expression is tightly controlled and induction of classical HIF target genes is primarily dictated by the action of the PHDs. Thus, HIF activity in general inversely matches PHD activity. (Schofield and Ratcliffe 2005) The inhibitory hydroxylation by FIH signifies another threshold for proper HIF activity but does not *per se* dictate the fate of the transcription factor. Interestingly, the destabilizing hydroxylation by the PHDs and the inhibitory hydroxylation by FIH are separated by the respective enzyme activities, possibly resulting in a gap with stable but partially inhibited HIFα (owing to FIH`s hydroxylation of the C-terminal trans-activation domain). Therefore, a small set of HIF target genes might not be regulated by inhibition of the C-terminal trans-activation domain - stable HIFα might be sufficient. Attempts to group HIF-target genes depending on the N-terminal trans-

activation domain or the C-terminal trans-activation domain have been reported. (Dayan *et al.* 2006) According, the FIH-restricted C-terminal trans-activation domain could promote HIF-target gene induction upon strong hypoxic insults, only.

In contrary, the N-terminal trans-activation domain might serve to promote transcription of a subset of genes by only moderately responding to changes in pO_2 (i.e. igfbp). (Dayan *et al.* 2006) Thus, it rather maintains the basal transcription of target genes. In this case stabilized HIFα would be important under normoxic conditions as well.

3.2.7 Screening for cadidate genes possible provoking the observed etoposide sensitivity

The knock-out of HIFα was expected to abrogate the response to hypoxia of numerous target genes. Therefore, the resulting etoposide sensitivity of the mutant MEF-*Hif1a*$^{-/-}$ cell line could have its origin in at least one of several possible pathways affected. In order to narrow down the list of possible candidates other assumptions had to be taken into account.

Candidate genes that might be responsible for the observed etoposide sensitivity were assumed to be involved in double-strand break repair or to participate in the repair of double-strand breaks. Two arguments led to this assumption.

First, the mutant MEF-*Hif1a*$^{-/-}$ cell line responded only to double-strand-breaking drugs but not to other toxins differentially then the wild-type cell line MEF-*Hif1a*$^{+/+}$.

Second, a plasmid rejoining assay had been published, showing that the MEF-*Hif1a*$^{+/+}$ wild-type cell line was able to re-ligate transfected, linearized plasmids to a much higher extent than the mutant MEF-*Hif1a*$^{-/-}$ cell line.(Unruh *et al.* 2003)

Dealing with a transcription factor, the etoposide tolerance of the MEF-*Hif1a*$^{+/+}$ wild-type cell line was expected to derive from a differential transcriptional gene regulation. HIF-dependent gene regulation under hypoxia leads to a pronounced mRNA induction for wild-type MEF-*Hif1a*$^{+/+}$ but not mutant MEF-*Hif1a*$^{-/-}$ for classical HIF-target genes. When comparing mRNA levels of wild-

type MEF-*Hif1a*$^{+/+}$ with mutant MEF-*Hif1a*$^{-/-}$ the difference in mRNA concentration for a classical HIF-target gene becomes much more pronounced upon hypoxic exposure but is minimal for normoxic “basla level” conditions. Opposite to classical HIF-target genes, the candidate gene(s) would only moderately respond to hypoxic induction. However, display a “basal level” difference in the mRNA concentration, even under normoxic conditions, when comparing wild-type MEF-*Hif1a*$^{+/+}$ with mutant MEF-*Hif1a*$^{-/-}$.

3.3 Non-homolgous end-joining and HIF

3.3.1 DNA-PKcs a key player in non-homologous end-joining

A candidate screen for double-strand break repair factors revealed a “basal level” difference in mRNA concentrations for DNA-PKcs, which did not respond to changes in the available oxygen concentration. Moreover, the difference in DNA-PKcs mRNA led us to measure the protein levels which were even more lowered in the mutant MEF-*Hif1a*$^{-/-}$ cell line. Due to its important function in end-joining, DNA-PKcs likely contributes to the remarkable etoposide sensitivity in mutant MEF-*Hif1a*$^{-/-}$ cells. In addition, low DNA-PKcs expression levels correlate with etoposide and IR sensitivity, (Jeggo *et al.* 1989; Caldecott *et al.* 1990; Blunt *et al.* 1995; Lees-Miller *et al.* 1995; Finnie *et al.* 1996; Taccioli *et al.* 1998; Johnson and Jones 1999; Smith and Jackson 1999; Boulton *et al.* 2000; Anderson *et al.* 2001; Belenkov *et al.* 2002; Eriksson *et al.* 2002; Lees-Miller and Meek 2003) (Gao *et al.* 1998; Willmore *et al.* 2004; Hardcastle *et al.* 2005; Zhao *et al.* 2006) whereas high levels of DNA-PKcs confer resistance to double strand inducing agents..(Tanaka *et al.* 1993; Shen *et al.* 1998; Hansen *et al.* 2003)

3.3.2 Wild-type MEF-Hif1a$^{+/+}$ differ from mutant MEF-*Hif1a*$^{-/-}$ in the number of double-strand breaks after genotoxic insult

Additional mechanisms might contribute to drug resistance in wild-type MEF-*Hif1a*$^{+/+}$. Accumulation of etoposide due to different cell cycle regulation or different detoxification of the interior milieu of the cell would also result in increased clonogenic survival of the MEF-*Hif1a*$^{+/+}$. However, detoxification of such different genotoxic stimuli as etoposide and the radicals formed by

ionizing radiation (IR) is unlikely to happen by similar means without resulting in similar patterns for other toxins (DFX, CPX, topoisomerase I inhibitor SN38). (Unruh *et al.* 2003) Anyhow, cell cycle regulation of the two cell lines (wild-type MEF-*Hif1a*$^{+/+}$ and mutant MEF-*Hif1a*$^{-/-}$) appears to be identical since cell doubling time and cell cycle distribution (as determined by fluorescence activated cell scanner (FACS) analysis) are comparable. Also etoposide induced cell cycle-arrest appears similar for both cell lines, wild-type MEF-*Hif1a*$^{+/+}$ and mutant MEF-*Hif1a*$^{-/-}$, as reflected by a simultaneous redistribution of the cell cycle profile after exposure to the toxin, further underscoring the uniformity in cell cycle regulation of the two cell lines.

However, differences become apparent in the amount of H2AX phosphorylation after genotoxic insult as detected by immuno-staining for γH2AX. ATM and ATR the only two kinases, apart from DNA-PKcs, known to phosphorylate H2AX in the course of DNA double-strand breaks, were checked for differential activity in the wild-type MEF-*Hif1a*$^{+/+}$ and mutant MEF-*Hif1a*$^{-/-}$ cell lines. It is likely that elevated ATM or ATR activity could result in etoposide sensitivity due to the role of ATM and ATR in mediating DNA repair and cell cycle control. Yet, after etoposide exposure, known ATM or ATR targets (Chk2 or Chk1, respectively) are phosphorylated equally well in wild-type MEF-*Hif1a*$^{+/+}$ as for mutant MEF-*Hif1a*$^{-/-}$. Therefore, the quantitative higher extent of H2AX phosphorylation after topoisomerase II inhibition is not based on differences in the activity of the kinases ATM or ATR. However, the higher sensitivity towards double-strand break-inducing agents of the mutant MEF-*Hif1a*$^{-/-}$ cells correlates with a larger degree of etoposide-induced H2AX phosphorylation of the mutant MEF-*Hif1a*$^{-/-}$ cell line when compared to the wild-type MEF-*Hif1a*$^{+/+}$ cell line. In addition, after etoposide administration the higher number of strand breaks as determined by comet assays also correlates with higher etoposide sensitivity and increased γH2AX for the mutant MEF-*Hif1a*$^{-/-}$ vs. wild-type MEF-*Hif1a*$^{+/+}$ cells. Thus, the amount of double-strand breaks after genotoxic insult differs from mutant MEF-*Hif1a*$^{-/-}$ to wild-type MEF-*Hif1a*$^{+/+}$ and is paralleled by etoposide sensitivity and low DNA-PKcs concentration.

3.3.3 Not only DNA-PKcs but also Ku80 transcription is lower in the mutant MEF-*Hif1a*$^{-/-}$ cells

Though possible that mutations in the DNA-PKcs coding or promoter sequence are responsible for the observed etoposide sensitivity of mutant MEF-*Hif1a*$^{-/-}$ cells, this appears unlikely since other genes showed a similar expression pattern as DNA-PKcs. Also the Ku80 protein levels were clearly lower in the mutant MEF-*Hif1a*$^{-/-}$ cells. Interestingly, the protein but not the mRNA levels of Ku70 were also decreased. However, the protein levels of the Ku-heterodimers were reported to be interdependent due to structural stabilization. (Downs and Jackson 2004) Therefore, HIF-deficiency affects the expression levels of several genes. Not only the induction of HIF-target genes is missing, but in the mutant MEF-*Hif1a*$^{-/-}$ cell line also the "basal levels" of a range of downstream genes are affected. This might be further supported by DNA microarray data. Data published include a set of genes with "basal level" differences even under normoxic conditions for HIF-1α-containing vs. HIF-1α-deficient mouse fibroblasts, possibly indicating a role for basic levels of HIF. (Greijer *et al.* 2005)

Under pathological conditions (i.e. cancer) basic levels of HIFα might be higher and the complex network of HIF- and hypoxia-dependent gene regulation disturbed. There is a set of HIF- and hypoxia-responsive factors which are repressors of the e-cadherin promoter.(Krishnamachary *et al.* 2006) Experiments with dominant-negative HIF-1α identified the transcriptional repressors (TCF3, Slug and Snail) and their target e-cadherin as downstream regulated genes of HIF-1α. Pathologically up-regulated HIF-1α contributes under normoxia to elevated mRNA expression levels of the repressors (TCF3, Slug and Snail) and therefore e-cadherin mRNA suppression. Paradoxically, under hypoxic conditions mRNA levels of the repressors (TCF3, Slug and Snail) are HIF-independently decreased and e-cadherin expression induced. Even though the transcription of the repressors (TCF3, Slug and Snail) does apparently not respond to hypoxia-induced HIF-1α levels, basic level of HIF-1α contributes to the "basal level" regulation of the repressors (TCF3, Slug and Snail) and therefore to the regulation of e-cadherin - especially in normoxia. Thus, HIF-1α might impact on the "basal level" of several genes

(i.e. by means of secondary factors) resulting in a complex HIF-dependent network, as exemplified for e-cadherin.

3.3.4 HIF deficiency and etoposide sensitivity

In order to confirm the HIF-dependent nature of our observation, HIF-1α was stably down-regulated by shRNA transfection. However, probably due to incomplete down-regulation of HIF-1α, shRNA-clones were not more sensitive to etoposide than the parental cell line. This, however, implies that already very low basic levels of HIF-1α suffice to confer etoposide resistance. Likewise, luciferase reporter activity (irrespective of mouse or human DNA-PKcs promoter) did not respond to increasing amounts of co-transfected HIF-1α in HeLa cells. Also the reintroduction of HIF-1α in MEF-*Hif1a*$^{-/-}$ cells did not affect luciferase activity of co-transfected reporter construct with the DNA-PKcs promoter. Even though two putative HIF-binding sites were found in the DNA-PKcs promoter, transcriptional activity of the DNA-PKcs promoter does apparently neither respond to transiently variable basic levels of HIF-1α nor transient presence of HIF-1α in MEF-*Hif1a*$^{-/-}$ cells. However, the relative luciferase activity of a transfected reporter construct with the DNA-PKcs promoter was substantially higher in the wild-type MEF-*Hif1a*$^{+/+}$ - consistent with a higher endogenous DNA-PKcs mRNA expression, elevated DNA-PKcs protein levels, decreased γH2AX-foci number after exposure to etoposide and enhanced resistance to double-strand break-inducing agents of the wild-type MEF-*Hif1a*$^{+/+}$ cells when compared to the MEF-*Hif1a*$^{-/-}$ cells. Though a direct transcriptional effect on the DNA-PKcs promoter is possible rather an indirect HIF-1 dependent effect might result in lower DNA-PKcs expression in the MEF-*Hif1a*$^{-/-}$ cells. Moreover, transient re-introduction of HIF-1α had no effect on DNA-PKcs expression indicative for a slow adaptation process that leads to change of DNA-PKcs promoter regulations.

3.3.5 Differences in the basic levels of HIF selects for different cell constitutions

The stable down-regulation of HIF-1α in the wild-type MEF-*Hif1a*$^{+/+}$ cell line did neither confer etoposide sensitivity nor affect DNA-PKcs expression. Not

even did the transient re-introduction of HIF-1α in mutant MEF-*Hif1a*$^{-/-}$ cells activate a co-transfected luciferase reporter with the DNA-PKcs promoter. Thus, the decrease in DNA-PKcs and the higher sensitivity to double-strand break inducers may only become evident when basic levels of HIF-1α are profoundly down-regulated for an extended period of time. These are exactly the conditions present in our MEF-*Hif1a*$^{-/-}$ model system where the levels of HIF-1α are maximally decreased (with no HIF-1α at all) for eternity.

Therefore, erasing HIF-1α from the cellular pool of regulatory factors may select for other cellular constitutions and thereby impact on the nicely balanced networks of factors involved in maintaining cell homeostasis. This result in a reorganization of regulatory networks to compensate for the lack of basic levels of HIF-1α. But why are most of the regulatory circuits in the MEF-*Hif1a*$^{-/-}$ not completely deregulated when compared to the wild-type MEF-*Hif1a*$^{+/+}$? Indeed, the uniformity of the two cell lines is striking, especially regarding the fact that HIF-1α is important for numerous cellular functions and is involved in the fine-tuning of several major pathways.(Wenger *et al.* 2005; Gordan *et al.* 2007; Kaidi *et al.* 2007)

However, the number of newly emanating cellular structures is limited. In order to cover for missing HIF-1α some cells will restructure their regulatory networks into inappropriate compositions for cellular homeostasis and thus collapse. Moreover, adaptations are probably only possible in a narrow range without disturbance of too many cellular processes (that is risk to loose control over cellular homeostasis).(Lovelock 1979) Maintenance of cell integrity, therefore, leads to mutant MEF-*Hif1a*$^{-/-}$ cells with reorganized but nearly identical regulatory networks to wild-type MEF-*Hif1a*$^{+/+}$ cells under standard cell culture conditions. Differences between the cell lines, wild-type MEF-*Hif1a*$^{+/+}$ and mutant MEF-*Hif1a*$^{-/-}$, will be manifested only under conditions of stress i.e. hypoxia.

There are, however, changes owing to the reorganization of regulatory networks (or redistribution of regulatory factors to cover up for HIF-1α) which set conditions different to the original cell status. In particular factors which do *per se* not influence cell homeostasis may be affected to changes. In their case the selective pressures to accurately compensate for devoid basic levels

of HIF-1α does not play a role. Since there is no influence on important cellular processes their deregulation does not jeopardize cell integrity under standard conditions, thus selection is not possible and even heavy deregulation will not end in a cellular break-down. These factors thus respond by adopting the new cellular settings, provoked by the absence of HIF-1α, without restraint. Therefore they may appear deregulated in mutant MEF-*Hif1a*$^{-/-}$ in relation to the wild-type MEF-*Hif1a*$^{+/+}$ cells, even under standard cell culture conditions, however without consequence for cellular continuity.

Although deregulated cells have to rely on such factors under special conditions i.e. stress after genotoxic insult. After stimulation with an appropriate stress the deregulated factors become now active, resulting in a different response of wild-type MEF-*Hif1a*$^{+/+}$ and mutant MEF-*Hif1a*$^{-/-}$ cell line. Thus, in addition to a different response to a hypoxic insult, other stress stimuli are likely to result in a different phenotype for wild-type MEF-*Hif1a*$^{+/+}$ cells compared to mutant MEF-*Hif1a*$^{-/-}$ cells. This may be the case for DNA-PKcs and the resulting etoposide sensitivity of mutant MEF-*Hif1a*$^{-/-}$. Summarized, the lack of basic levels of HIF-1α leads to aberrant cellular compositions which are the base for a differing reaction on stress stimuli. On the other hand also cells with elevated basic levels of HIF-1α may result in aberrant cellular compositions and yet other phenotypes. Thus, anomalous basic levels of HIF-1α, as arising in cancerous tissue, spawns cells with different reaction on stress stimuli. Targeting such cells as in the case of cancer therapy then will select for more resistant clones and thereby poorer clinical outcome of tumor therapy.

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5 Appendix

5.1 Table of figures

5.1.1 Introduction

5.1.2 Determination and modulation of prolyl-4-hydroxylase domain (PHD) oxygen sensor activity

5.1.3 Increased prolyl-4-hydroxylase domain (PHD) proteins compensate for decreased oxygen levels: evidence for an autoregulatory oxygen sensing system

5.1.4 Hypoxia-inducible factor-dependent DNA double-strand break repair contributes to tumor cell chemoresistance

5.1.5 Induction of the HIF system by low levels of HSP90 inhibitors

5.1.6 Unpublished data

5.2 Contributions

I contributed to the following publications / manuscripts as indicated:

5.2.1 Determination and modulation of prolyl-4-hydroxylase domain (PHD) oxygen sensor activity

Figures 1 / 3 / 4

Protein purification leading to figure 2B / C

Establishing purification of wild-type GST-HIF-2αODD and initial setup of the assay conditions for figure 5

5.2.2 Increased prolyl-4-hydroxylase domain (PHD) proteins compensate for decreased oxygen levels: evidence for an autoregulatory oxygen sensing system

Setup of cell culture, infection and purification conditions leading to purified recombinant GST-PHD2 and GST-PHD3 from baculoviral infected Sf9 insect for figure 7

5.2.3 Hypoxia-inducible factor-dependent DNA double-strand repair contributes to tumor cell chemoresistance

Figures 1 / 3 / 4 / 5A / 5B

Establishment of extraction method and immunoblot conditions for figure 2C

Initial experiments leading to figure 2D

5.2.4 Induction of the HIF system by low levels of HSP90 inhibitors

RT-qPCR quantification of mRNA levels for CAIX, PHD2 and PHD3 leading to figure 5

5.3 Curriculum Vitae

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Education

since 2004	**PhD thesis**, Group of Prof. Dr. R.H. Wenger, Institute of Physiology, University of Zurich
1999-2004	**Master of science ETH** (Biochemistry), Swiss Federal Institute of Technology, (ETH) Zurich
1993-1998	**Matura**, Alte Kantonsschule Aarau, type C (mathematics)
1994-1995	Lycée de Notre-Dame de Kerbertrand (France)

Work experience

PhD thesis
"Oxygen-dependent regulation of Hypoxia-inducible factor (HIF) and its role in cancer therapy resistance", Institute of Physiology, University of Zurich

Master thesis
"Protein purification of epoxide hydrolase of *Sphingomonas* sp. HXN-200", Institute of biotechnology, ETH Zurich

Term paper
"Biocatalysis of substrates", Institute of biotechnology, ETH Zurich

Practical courses
-Course in Immunology
-Biological Chemistry: directed protein evolution
-Biochemical and molecular-biological course
-Organic Chemistry, Physical Chemistry, Inorganic Chemistry

Teaching
Assistance of practical courses for human biology and medical students (2004-2007)

Languages
German (native), English (fluent), French (fluent)

5.4 Posters and Presentations

09. 2006 Cancer Research Retreat, Monte Verità, Ascona: Presentation
10. 2006 Annual Meeting of the Swiss Physiological Society. Fribourg (CH): Short Presentation and Poster
11. 2006 Institute Seminar, Zurich: Presentation
02. 2007 Euroxy Course, Maastricht (NE): Presentation and Poster

5.5 Publication List

Wirthner R., Wrann S., Wenger R.H and Stiehl D.P Hypoxia-inducible factor-dependent DNA double-strand break repair contributes to tumor cell chemoresistance
Submitted

Wirthner R., Balamurugan K., Stiehl D.P., Barth S., Spielmann P., Oehme F., Flamme I., Katschinski D.M., Wenger R.H. and Camenisch G. Determination and Modulation of Prolyl-4-Hydroxylase Domain Oxygen Sensor Activity
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Chem Commun. **2003**, 8: 960-61

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